GLYCOPROTEINS IN COLON CANCER: POSSIBLE ROLE OF TUMOR ASSOCIATED ANTIGEN 90K

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By

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ABSTRACT

One of the most consistent biochemical changes associated with colon cancer progression is the altered expression of cell-associated carbohydrates. For example, the elevated expression of β1-6 branched N-linked oligosaccharides correlates with the presence of metastatic disease in colon cancer patients. Thus, it has become desirable to identify glycoproteins that are modified by these cancer-associated carbohydrates. Previous work in our laboratory identified the tumor-associated antigen 90 kDa (TAA90K) as a carrier of these cancer-associated carbohydrates. Since TAA90K has been previously implicated in cancer progression and metastasis, we examined its expression and function in human colon tumors. Immunohistochemical analysis revealed elevated expression of TAA90K in all tumor samples analyzed compared to normal colon. To examine the function of TAA90K in colon cancer, we performed protein binding and cellular assays with TAA90K purified from HT-29 human colon cancer cells infected with recombinant vaccinia virus expressing TAA90K. Purified TAA90K bound to ECM proteins including fibronectin, collagen IV, laminins-1, -5 and -10 and galectin-3. Unlike TAA90K isolated from other cell types, TAA90K isolated from HT-29 cells failed to mediate adhesion of colon cancer and normal cell lines, due in part to cell-specific glycosylation differences. Although TAA90K did not directly mediate cellular adhesion, it did modulate galectin-3-dependent adhesion of HT-29 cells. In addition, TAA90K bound to and was a substrate for MMP-7, a matrix metalloproteinase previously implicated in colon cancer progression. MMP-7-cleavage of TAA90K had little effect on its binding to pro- and active MMP-7, laminin-1 and galectin-3, but reduced significantly its binding to fibronectin and laminin-10. In addition, treatment of cells with MMP-7-cleaved TAA90K resulted in lower levels of proMMP-7 in the conditioned medium than cells treated with intact TAA90K. This may be mediated by the reduced binding of MMP-7-cleaved TAA90K to IL-6 and IL-1β, cytokines previously implicated in enhanced proMMP-7 expression in prostate cancer cells. Thus, a possible mechanism by which TAA90K may contribute to colon cancer progression is by modulating tumor cell adhesion to extracellular proteins and extracellular matrix remodeling through interactions with MMP-7 and galectin-3.

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DEDICATION

To my husband Jason. You are the love of my life. Thank you for putting your dreams on hold so that I could complete mine. I couldn't have done it without you.

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ABBREVIATIONS

ACF	aberrant crypt foci
ADAMTS	a disintigrin and metalloproteinase with thrombospondin motifs
AP	alkaline-phosphatase
APC	adenomatous polyposis coli
APMA	p-aminophenylmercuric acetate
Asn	asparagine
β1-6 branch	[GlcNAcβ1-6Manα1-6Manβ]
BCIP	5-bromo-4-chloro-3-indolyl-phosphate-toluidine
BSA	bovine serum albumin
BTB/POZ	*B*road complex, *t*ramtrack, *b*ric a brac/*po*xvirus and *z*inc finger
BUdR	5-bromodeoxyuridine
CA	carbohydrate antigen
CEA	carcinoembryonic antigen
CIMP	CpG island methylator phenotype
CMF-PBS	calcium and magnesium free-phosphate buffered saline
CRD	carbohydrate recognition domain
CyCAP	cyclophilin-C associated protein
DCBE	double-contrast barium enema
DCC	deleted in colon cancer
DMEM	Dulbecco's minimum essential medium
DMJ	deoxymannojirimycin
DMSO	dimethylsulfoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis coli
FasL	Fas ligand
FBS	fetal bovine serum
FOBT	fecal-occult blood test
Fuc	fucose
GDP	guanidine diphosphate
Glc	glucose
GlcNAc	N-acetylglucosamine
Gal	galactose
GalNAc	N-acetylgalactosamine
GnT	N-acetylglucosaminyltransferase
HNPCC	hereditary non-polyposis colorectal cancer
HPV	human papillomaviruses
HUVEC	human umbilical vein endothelial cells
ICAM	intercellular adhesion molecule

IF	immunofluorescence
IFN	interferon
Ig	immunoglobulin
IPTG	isopropylthiogalactoside
IRF-E	interferon responsive element
LAK	lymphokine-activated killer cells
LAMP	lysosomal membrane associated protein
Le^a	Lewis A
Le^x	Lewis X
Le^y	
L-PHA	leucophytohemagglutinin
LPS	lipopolysaccharide, endotoxin
MAb	monoclonal antibody
Man	mannose
MAPK	mitogen-activated protein kinase
Mgat5	mannoside acetyl glucosaminyl transferase 5
MMP	matrix metalloproteinase
MMR	mismatch repair genes
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide: thiazolyl blue
NaN_3	sodium azide
NBT	p-nitroblue tetrazidium chloride
N-glycans	asparagine-linked oligosaccharides
NK	natural killer cells
NMS	normal mouse serum
NSCLC	non-small cell lung carcinoma
O-glycan	serine/threonine-linked oligosaccharides
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PI3K	phosphatidyl inositol 3-kinase
PMSF	phenylmethylsulfonylfluoride
P-P-Dol	pyrophosphoryldolichol
PVDF	polyvinylidene fluoride
RGD	asparagine-glycine-aspartate
pRB	retinoblastoma protein
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	serine
SF media	serum free media
Sialyl T	sialyl transferase
sLe^x	sialyl-Lewis X
sLe^a	sialyl-Lewis A
SRCR	scavenger receptor cysteine rich
STAT	signal transducer and activator of transcription
STn	sialyl-Tn antigen
Sw	swainsonine

TAA90K	tumor-associated antigen 90 kDa
TBS	Tris-buffered saline
TCF	T cell factor
TE	trypsin-EDTA
TGF	transforming growth factor
Thr	threonine
TIMP	tissue inhibitor of matrix metalloproteinases
TK	thymidine kinase
TNF	tumor necrosis factor
TMN	tumor, lymph node metastasis, distant metastasis classification
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WGA	wheat germ agglutinin

1.0 INTRODUCTION

Cancer is a widespread disease accounting for millions of deaths each year. Cancer is a complex disease due to its ability to arise in a number of different tissues and organs. Treatment and diagnosis of cancer can be difficult due to the large number of mutations or alterations that can arise during initiation, progression and invasion. Cancer research focuses on identifying cancer-associated alterations in cells that may contribute to cancer progression.

The research presented in this thesis focuses on the tumor-associated antigen 90 kDa (TAA90K), a glycoprotein which bears cancer-associated carbohydrates and has been associated with the progression of a number of cancers.

2.0 LITERATURE SURVEY

2.1 CANCER

Cancer represents a major global health problem accounting for 7 million deaths worldwide in 2001 (Danaei *et al.*, 2005). Analysis of trends in cancer statistics led to a prediction for 2005 that almost 1.4 million new cancer cases would result in over half a million deaths in the United States alone. These findings indicate that almost 1 in 4 deaths in the United States is cancer-related. As a result, cancer is ranked as the second leading cause of all deaths, behind heart disease. The most commonly diagnosed types of cancer among men include prostate, lung and colorectal cancer whereas cancers of the breast, lung and colorectum predominate in women (Jemal *et al.,* 2005). Cancer results in the development of a tumor at a primary site, however it is the metastatic spread of cancer cells to secondary sites that is usually resistant to conventional therapies and accounts for over 90% of human cancer deaths (Weigelt *et al*., 2005). These statistics emphasize the need for improved methods of prevention, detection and treatment.

The development of cancer or carcinogenesis is initiated with mutations that confer upon a cell an altered phenotype. The aberrant cell has the potential to develop into a mass of altered cells or a tumor. Mutations can be present in germline cells and

passed on from one generation to the next as is observed in hereditary types of cancer such as xeroderma pigmentosum and adenomatous polyposis coli. Alternatively, exposure of somatic cells to carcinogens, transforming viruses and other mutagens can induce mutations resulting in sporadic types of cancer. For example, it has been proposed that 35% of the 7 million worldwide cancer deaths in 2001 are attributable to a set of behavioural risk factors or exposure to environmental risk factors (Danaei *et al.,* 2005). For example, overweight or obese individuals have an elevated body mass index which is a risk factor for colorectal cancers and post-menopausal breast cancer. Similarly a lifestyle consisting of physical inactivity and a diet reduced in fruits and vegetables were also risk factors for colorectal cancer. On the other hand, cigarette smoke contains known carcinogens, which are risk factors for cancers of the lung, mouth, stomach and liver. Exposure to environmental factors such as urban air pollution was also attributable to lung cancer. Elevated consumption of alcohol was shown to be a behavioural risk factor for liver, mouth, and breast cancer. Other behavioural risk factors include exposure to viruses such as certain strains of human papilloma virus spread by sexual contact or to hepatitis viruses B or C, which can be acquired by using contaminated needles. Transforming viral proteins expressed by human papilloma virus or hepatitis viruses have been linked to cervical cancer and liver cancer, respectively (Danaei *et al.,* 2005).

2.1.1 Initiation

The development of a tumor or tumorigenesis is initiated when genetic or epigenetic modifications affect the activity of one of three types of genes, namely oncogenes, tumor suppressor genes or stability genes (reviewed in Vogelstein and Kinzler, 2004). Genetic mutations in proto-oncogenes result in enhanced activity of the oncogene product compared to the wild-type gene product. For example, chromosomal translocations, gene amplification or intragenic mutations that affect the activity of the gene product are all mechanisms by which oncogenes become activated (reviewed in Vogelstein and Kinzler, 2004). Oncogene products that are involved in cellular signaling events result in the enhanced transcription of gene products responsible for cellular proliferation. For example, a constitutively active *ras* oncogene product

enhances signaling of the Ras-Raf-MAP (mitogen activated protein) kinase pathway which results in activation of transcription factors such as Myc, Fos or Jun, all of which transcribe gene products necessary for enhancing the rate of cellular proliferation (Medema and Bos, 1993). Mutations in the *ras* oncogene have been observed in 50% of human colon tumors (Kinzler and Vogelstein, 1996).

Tumor suppressor genes normally play a role in the regulation of cellular homeostasis by monitoring cell cycle regulation and DNA damage. For example, the retinoblastoma protein (pRb) has the ability to block cellular proliferation by preventing aberrant cells from entering the S phase of the cell cycle (Weinberg, 1995). Another tumor suppressor, p53, becomes activated in response to DNA damage and plays a role in cell cycle arrest and the induction of apoptosis (Prives and Hall, 1999). Mutations in tumor suppressor genes induced by missense mutations, truncations or deletions can result in a gene product that is inactive or has reduced activity. Reduced activity of tumor suppressors allows aberrant cells to continue through the cell cycle, resulting in synthesis of mutated DNA and the accumulation of a mass of abnormal cells. For example, the functional inactivation of p53 is observed in 50% of all cancers (Harris, 1996).

The final class of genes frequently mutated in cancer consists of the stability genes. Stability genes include mismatch repair genes, nucleotide-excision repair genes and base-excision repair genes. Collectively, these genes are responsible for repairing DNA damage incurred by normal DNA replication or by exposure to DNA damaging agents. In addition, they play a role in homologous recombination and chromosomal segregation (reviewed in Vogelstein and Kinzler 2004). Analysis of the number of spontaneous mutations observed in normal cells does not account for the large numbers of mutations observed in human cancers. Therefore, cancer cells are hypothesized to display a mutator phenotype (reviewed in Loeb, 2001). This mutator phenotype results when stability genes are lost and genomic instability arises. This phenotype is characterized by chromosomal instability and microsatellite instability. Microsatellite instability occurs when mutations arise in highly repetitive regions of DNA, resulting in the inactivation or mutation of mismatch repair genes. Chromosomal instability refers to mutations that affect chromosomal segregation resulting in a change in the number of

chromosomes, known as aneuploidy (reviewed in Loeb, 2001). The inactivation of stability genes results in the accumulation of mutations at an elevated rate compared to the mutation frequency observed in normal cells (reviewed in Vogelstein and Kinzler, 2004).

Although genetic mutations play an important role in the development of cancer, epigenetic changes can also result in altered expression of gene products (reviewed in Kondo and Issa, 2004). Epigenetic changes include alterations to DNA or chromatin such as DNA methylation or histone modification that affect the accessibility of transcription factors and accessory proteins to the promoter regions of certain genes (Bird and Wolffe, 1999). The DNA in normal cells is frequently methylated, resulting in the addition of a methyl group to the 5' cytosine of CpG dinucleotides. DNA hypermethylation has been observed in a number of cancers and is characterized by elevated levels of DNA methylation compared to normal cells (reviewed in Kondo and Issa, 2004). This aberrant DNA methylation results in the silencing of tumor suppressor genes such as pRb resulting in reduced gene expression and the perpetuation of a cancer phenotype (Santini *et al.,* 2001). Other examples of epigenetic changes common to cancer cells are histone modifications. Histones play a role in the condensation of DNA by packaging it into nucleosomes. Histone proteins can be modified by phosphorylation, acetylation and methylation, all of which can affect the packaging of DNA into nucleosomes, which in turn affects the accessibility of transcription factors and accessory proteins to promoter regions (Jenuwein and Allis, 2001). Thus, histone modifications and DNA methylation have the ability to affect the expression of genes without altering the DNA sequence.

2.1.2 Characteristics of Cancer Cells

The accumulation of both genetic mutations and epigenetic changes results in transformed cells, which are characteristically distinct from their normal progenitor cells, allowing for progression of tumorigenesis (reviewed in Hanahan and Weinberg, 2000). For example, normal cells respond to external stimuli in a paracrine manner through cell surface receptors that bind growth factors secreted by other types of cells. In contrast, cancer cells acquire the ability to act in an autocrine manner by secreting

growth factors and effectively regulating their own growth and proliferation (Fedi *et al*., 1997). In addition, cancer cells can also enhance their rate of proliferation by increasing the expression of cell surface receptors that bind to growth factors, resulting in enhanced sensitivity to external stimuli. Alternatively, cancer cells may produce mutated growth factor receptors that are constitutively active and stimulate intracellular signals even in the absence of growth factor binding to the receptor (Fedi *et al*., 1997).

Another difference between cancer cells and normal cells is their response to antigrowth signals (reviewed in Hanahan and Weinberg, 2000). Normally, cellular proliferation can be halted and cells forced into a quiescent state by the binding of external antigrowth factors to their cell surface receptors. For example, transforming growth factor β (TGFβ) binds to its cell surface receptor (TGFβR) and signaling events result in the activation of pRb, effectively blocking cellular proliferation. In some types of cancer, decreased expression of the TGFβR, expression of a mutant receptor lacking a ligand binding domain or expression of a mutant pRb can allow the cell to elude this checkpoint and continue to proliferate at an elevated rate (Fynan and Reiss, 1993), thus contributing to the progression of tumorigenesis.

Apoptosis is a process that normally functions as a protective mechanism to eliminate potentially aberrant cells from further proliferation (Kerr *et al*., 1972). Signaling events are initiated in response to DNA damage, hypoxia or loss of cell-cell or cell-extracellular matrix (ECM) contacts (Evan and Littlewood, 1998; Giancotti and Ruoslahti, 1999). One pathway leading to the initiation of apoptosis is mediated by the tumor suppressor protein p53, which becomes activated in response to cellular stress and functions to increase the expression of the pro-apoptotic protein Bax (Green and Reed, 1998). Elevated levels of Bax result in the release of cytochrome *c* from the mitochondria, which is necessary for the activation of a class of proteases known as caspases. These enzymes are responsible for the proteolysis of cellular organelles and the genome (Thornberry and Lazebnik, 1998). In contrast to normal cells, cancer cells have acquired mechanisms to evade apoptosis, thus enabling them to continue to proliferate even in the presence of aberrant genetic mutations thus enhancing tumor progression (reviewed in Hanahan and Weinberg, 2000). One mechanism that cancer

cells employ to achieve this is by expressing a p53 protein with reduced activity (Symonds *et al*., 1994).

Another characteristic cancer cells acquire that is important for the progression of tumorigenesis is their ability to undergo an unlimited number of cellular divisions (reviewed in Hanahan and Weinberg, 2000). Normal cells have a pre-determined number of cellular divisions before they stop dividing and enter senescence (reviewed in Hayflick, 1997). This limited replicative potential has been shown to be the result of shortening of the ends of chromosomes, or telomeres, following multiple rounds of DNA replication. Cancer cells overcome this limited replicative potential by upregulating the activity of telomerase, an enzyme that replicates the telomeres on the ends of chromosomes, effectively preventing telomere shortening (Bryan and Cech, 1999). This enhanced telomerase activity and subsequent telomere maintenance has been observed in virtually all types of malignant cells (Shay and Bacchetti, 1997).

The aforementioned characteristics of cancer cells confer upon cells the ability to replicate at an elevated rate. Although enhanced proliferation is necessary for tumorigenesis, the development of a tumor that is larger than 1 mm in diameter requires the development of new blood vessels to supply the tumor cells with adequate oxygen and nutrients. If a tumor exceeds 1 mm in diameter, cancer cells must acquire the ability to initiate angiogenesis (Bouck *et al*., 1996; Hanahan and Folkman, 1996). Angiogenesis is a process of neovascularization involving the development of new blood vessels. It is a necessary event during normal processes such as menstruation or embryogenesis and is regulated by both stimulatory and inhibitory signals (Hanahan and Folkman 1996). For example, vascular endothelial growth factor (VEGF) is a soluble factor that binds to its receptor, VEGFR, on the surface of endothelial cells, thus stimulating the development of new blood vessels (Fedi *et al.,* 1997). Angiogenesis by cancer cells involves invasion of the surrounding tissue by the secretion of proteases in addition to enhanced secretion of angiogenesis-stimulating factors that induce the migration of endothelial cells towards the tumor (Stetler-Stevenson, 1999).

The final characteristic of cancer cells is the ability to invade surrounding tissue and establish metastases at distant organs. Metastasis is characterized by the spread of cancer from a primary tumor to secondary sites and organs, representing the last step of

tumor progession (reviewed in Hanahan and Weinberg, 2000). There are two main hypotheses that attempt to explain how metastatic cells arise from the primary tumor. The first theory, known as the clonal progression model, hypothesizes that genetic alterations which would enhance the ability of cells within the primary tumor to metastasize may arise at any time during tumorigenesis. Cells with this metastatic phenotype would predominate in the cell population of the primary tumor (Nowell, 1976). An alternate theory, known as the rare variant model, hypothesizes that metastatic cells represent a minor population of the primary tumor arising from rare variants. This model is supported by experiments that isolated variants with distinct differences in metastatic potential from the cells making up the primary tumor (Fidler and Kripke, 1977; Fidler and Hart 1982). The primary tumor is thought to be heterogeneous in nature resulting from genetic instability that results during the process of tumorigenesis (Loeb, 2001). Thus, as tumorigenesis progresses, resulting tumors are heterogeneous, consisting of cells with varying ability to invade and metastasize (Fidler, 2002). Analysis performed by Kerbel (1990) revealed that rare metastatic variants preexist within the primary tumor, but may replicate to become the dominant cell type composing the tumor mass. This is referred to as the clonal dominance theory of cancer progression (Kerbel, 1990).

The process of tumorigenesis is not sufficient for metastasis. Further genetic mutations are required in order for cells to acquire metastatic potential. Cells with metastatic potential share some genomic similarities to the less metastatic cells comprising the primary tumor. However, examination of differences in gene expression between these two types of cells led to the discovery of a class of genes known as metastasis suppressor genes (van't Veer *et al*., 2002). The expression of these genes, including NM23 has been observed to be down-regulated in highly metastatic variants as compared to their less metastatic counterparts (reviewed in Steeg, 2003).

The process of metastasis is often referred to as the metastatic cascade. One of the first barriers for metastasis is for cells with metastatic potential to disengage from the primary tumor. This is accomplished by loss of cell-cell and cell-ECM contacts (reviewed in Hanahan and Weinberg, 2000). For example, tumor cells maintain cell-cell contacts with each other through interaction of cell surface receptors known as E-

cadherin receptors. Tumor cells with enhanced metastatic potential have been shown to express reduced or mutated E-cadherin receptors on their cell surfaces allowing them to separate from the primary tumor (Bracke *et al*., 1996). In addition, integrins are cell surface receptors that play a role in mediating cell-ECM interactions. Integrins bind to ECM components such as laminins, collagens and fibronectin. The interaction between integrin receptors and their ligands can initiate the transmission of signals to the inside of the cells. This integrin activation can also result in the lateral clustering of integrin receptors and their ECM ligands to form complexes that are linked to the cytoskeleton. These complexes are called focal adhesions and they are linked to actin filaments, which propel the cell during migration. Loss or altered expression of integrin receptors by tumor cells can result in reduced binding to ECM components in addition to altered intracellular signaling (reviewed in Giancotti and Ruoslahti, 1999).

Once cells have separated from the primary tumor they must invade through the surrounding stromal tissue, another barrier to metastasis. This process is mediated by enhanced secretion of proteases by invading cells and the surrounding stroma. Matrix metalloproteinases (MMPs) are a class of proteases that play an important role in invasion of metastatic cells. MMPs are represented by a family of approximately 26 members, all of which contain a prodomain and a catalytic domain (Figure 2.1). The catalytic domain consists of a conserved binding site for the catalytic zinc ion. Five family members reside on the cell surface as they have transmembrane domains and are referred to as membrane-type (MT)-MMPs. The other family members function extracellularly as they are secreted in zymogen form as proMMPs. MMPs are activated upon cleavage by other active MMPs or by other proteases such as plasmin or urokinase-type plasminogen activator. MMPs are important in normal processes such as wound repair, inflammation and embryogenesis (reviewed in Egeblad and Werb, 2002). However, the secretion of MMPs by both tumor cells and stromal tissues has been observed at the leading edge of invasive tumors. In fact, proMMPs and their activating

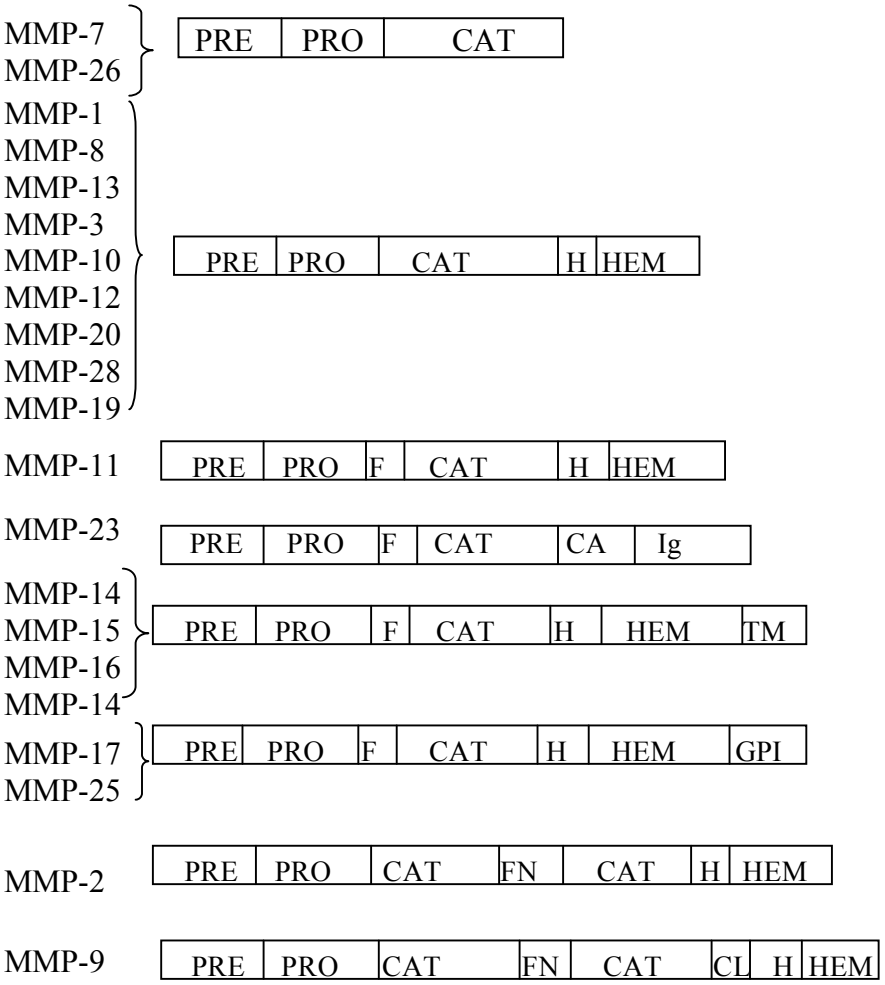


Figure 2.1: Matrix metalloproteinases and their putative domains. PRE, pre-domain; PRO, pro-domain; CAT, catalytic domain; H, hinge; HEM, hemopexin domain; F, furin-cleavage consensus domain; FN, fibronectin-like domain; GPI, glycophosphatidyl inositol anchor; TM, transmembrane domain; Ig, immunoglobulin-like domain; CA, cysteine array; CL, collagen-like domain. Adapted from Wagenaar-Miller *et al.* (2004).

factors are secreted by both the tumor and stromal cells, thus indicating that both cell types likely play a role in invasion (reviewed in Deryugina and Quigley, 2006).

In addition to playing a role in invasion of the ECM and stromal tissue, MMPs have been shown to play a role in a number of stages during metastasis (reviewed in Deryugina and Quigley, 2006). For example, remodeling of the ECM as well as cell surface receptors by MMP cleavage can alter cell-ECM as well as cell-cell contacts. MMP-3 has been shown to degrade E-cadherin on the surface of tumor cells, resulting in loss of tumor cell-tumor cell contacts (Veerle *et al.,* 2000). MMPs have also been shown to cleave cell surface receptor CD44, which binds to hyaluronic acid in the ECM. This cleavage results in loss of tumor cell-ECM contacts, allowing tumor cells to escape from the primary tumor (Nakamura *et al.,* 2004). Normally, the loss of cell-cell and cell-ECM contacts induces a type of apoptosis referred to as anoikis. There is some evidence to suggest that the expression and secretion of MMPs may protect liberated tumor cells from undergoing anoikis (reviewed Deryugina and Quigley, 2006).

MMPs have also been implicated in the process of intravasion and enhanced survival of tumor cells upon entry into the circulation. Intravasion is characterized by the entry of metastatic tumor cells into the blood or lymphatic vessels. Upon entry into the vasculature, tumor cells must survive attack by the immune system if they are to establish tumor foci at a secondary organ (reviewed in Deryungina and Quigley, 2006). MMPs secreted by tumor cells have been implicated in immunosuppression. Specifically, MMP-9 has been shown to mediate the cleavage of IL-2Rα, which results in the reduced infiltration of lymphocytes to the tumor cells (Sheu *et al.,* 2001). MMPs may also protect circulating tumor cells by enhancing embolization, which involves the establishment of tumor cell clusters with aggregated platelets (Alonso-Escolano *et al.,* 2004). For example, the activation of MMP-2 by membrane type-1 (MT1)-MMP present on the tumor cell surface results in the enhanced activation and subsequent aggregation of platelets with the tumor cells. Embolization of tumor cells with platelets may allow the tumor cells to survive for longer periods of time in the circulation compared to individual tumor cells (reviewed in Deryugina and Quigley, 2006).

Upon embolization, tumor cells move through the circulation until they arrest at the capillary bed of secondary organs. This process is mediated by cell adhesion and

involves the expression of cell surface receptors known as selectins, which mediate tumor cell-endothelial cell interactions. Adherence of the tumor emboli at secondary sites is also mediated by integrins, which establish tumor cell-ECM interactions (Hood and Cheresh 2002). Finally, extravasion occurs and tumor cells move through the blood vessel wall into the organ tissue and establish a tumor at this secondary location, finalizing the metastatic cascade. The growth of a tumor at a secondary site is dependent on the microenvironment of the secondary organ, which can influence cellular proliferation, gene expression and angiogenesis of the metastases (Chambers *et al*., 2002; Fidler, 2002; Fidler, 2003).

2.2 COLON CANCER

Colorectal cancer remains one of the most prevalent epithelial cancers, resulting in nearly 70, 000 deaths annually in North America (Weir *et al.,* 2003; Ellison and Gibbons, 2004). It is estimated that approximately half of the western population will develop a colorectal tumor by the age of 70 with 1 in 10 of theses cases leading to malignancy (Kinzler and Vogelstein, 1996). Colorectal cancer affects both the rectum and colon, which consists of the cecum, the ascending colon, the transverse colon, the descending colon and the sigmoid colon (Figure 2.2). The large intestine is composed of layers of tissue consisting of the epithelium, lamina propria, the muscularis mucosae and the submucosa. The epithelium layer consists of crypts that are composed of a number of different types of cells. Stem cells are located at the base of intestinal crypts and have the ability to differentiate into columnar epithelial cells, mucous cells or endocrine cells (Figure 2.3). Upon differentiation, cells migrate from the base of the crypt to the lumen of the large intestine. Columnar epithelial cells are found lining the lumen of the large intestine and are responsible for its absorptive function. These cells are frequently shed into the lumen of the intestine and continually replaced by the differentiating stem cells located at the crypt base. Mucous cells are secretory cells that produce, store and secrete mucous into the lumen of the large intestine. Finally, endocrine cells secrete hormones that are necessary for maintenance of intestinal cellular function (reviewed in Tejpar and Van Cutsem, 2002). The lamina propria is composed of connective tissue containing cells such as fibroblasts, lymphocytes and

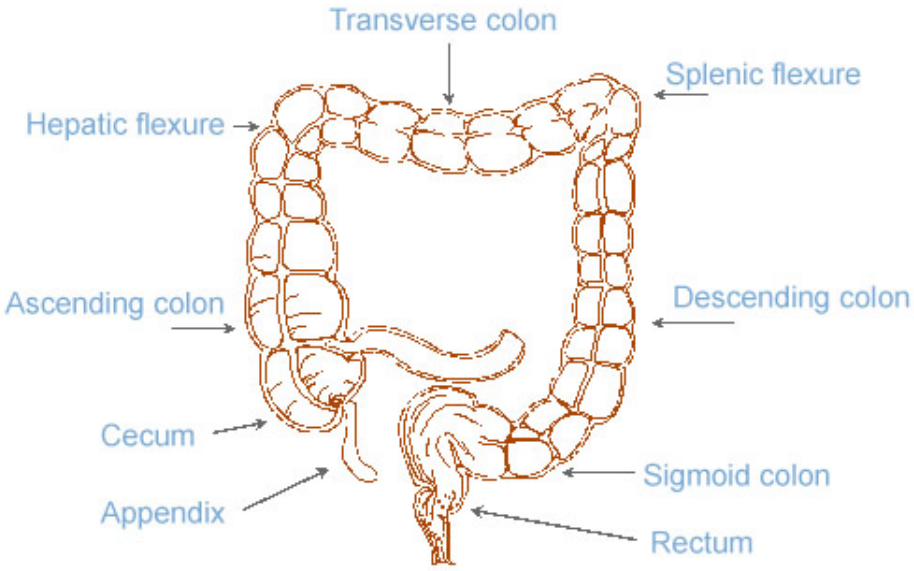


Figure 2.2: Anatomy of the human colon. Anatomy of the human colon and rectum indicating the subdivision into various segments. From Ponz de Leon and Di Gregorio (2001).

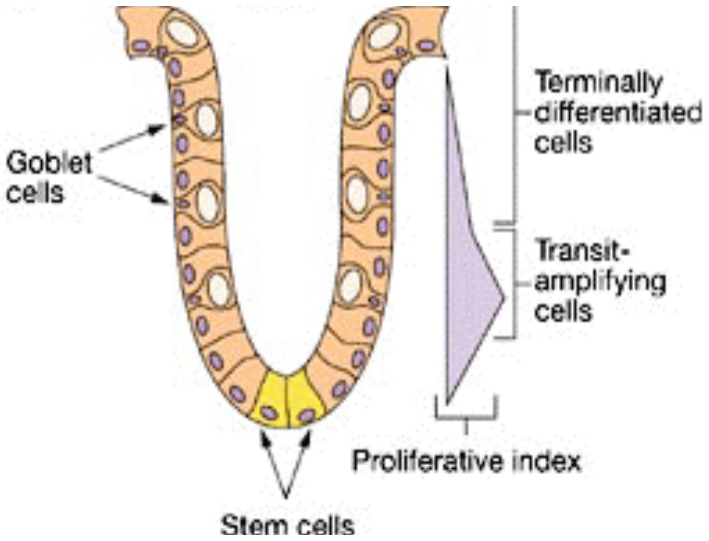


Figure 2.3: Structure of the colon. The cellular composition of the crypts, indicating the location of the stem cells making up the colon. From McDonald *et al*., 2006.

macrophages. It extends between the crypts and the muscularis mucosae, which is comprised of a thin layer of smooth muscle. The submucosa is similar in composition to the lamina propria, however it also contains vascular elements such as arterioles, venules and lymphatic vessels (reviewed in Ponz de Leon and Di Gregorio, 2001).

Colorectal cancer arises as a result of the accumulation of both genetic and epigenetic events that result in the transformation of normal colonic epithelium. The process of colorectal tumorigenesis proceeds according to well characterized and discreet morphological and genetic stages (Figure 2.4) (Fearon and Vogelstein, 1990).

2.2.1 Genetic and epigenetic modifications in sporadic and inherited colorectal cancers

Colorectal cancer can arise sporadically following alterations to somatic cells induced by endogenous factors such as DNA replication errors, reactive oxygen species that are generated by cellular metabolism and by exogenous factors such as exposure to carcinogens (reviewed in Tejpar and Van Cutsem, 2002). Colorectal cancer occurs following activation of oncogenes and inactivation of tumor suppressor genes and stability genes. One of the most common oncogenes activated during colorectal carcinogenesis is *ras* (Fearon and Vogelstein, 1990). As previously mentioned, activation of the *ras* oncogene product results in enhanced cellular proliferation allowing for the clonal expansion of aberrant cells. Inactivation of the tumor suppressor gene adenomatous polyposis coli (APC) is one of the most frequent alterations observed in colorectal cancer, present in over 80 % of colorectal carcinomas (Powell *et al.,* 1992). The wildtype APC protein binds to cytosolic β-catenin mediating its interaction with the cytoplasmic domain of E-cadherin resulting in the establishment of cell-cell contacts (Wang *et al*., 2006). APC also plays a role in regulating the cytoplasmic levels of β-catenin by targeting it for ubiquitination. When APC is mutated in cancer, the level of free cytosolic β-catenin is elevated resulting in its subsequent translocation to the nucleus and activation of transcription factors including T cell factor (TCF) transcription factors (Figure 2.5) (Molatore and Ranzani, 2004). These transcription factors are responsible for the transcription of genes that regulate cellular proliferation in addition to genes involved in the regulation of apoptosis, cellular invasion and chromosomal segregation (Molatore and Ranzani, 2004). Interestingly, the remaining

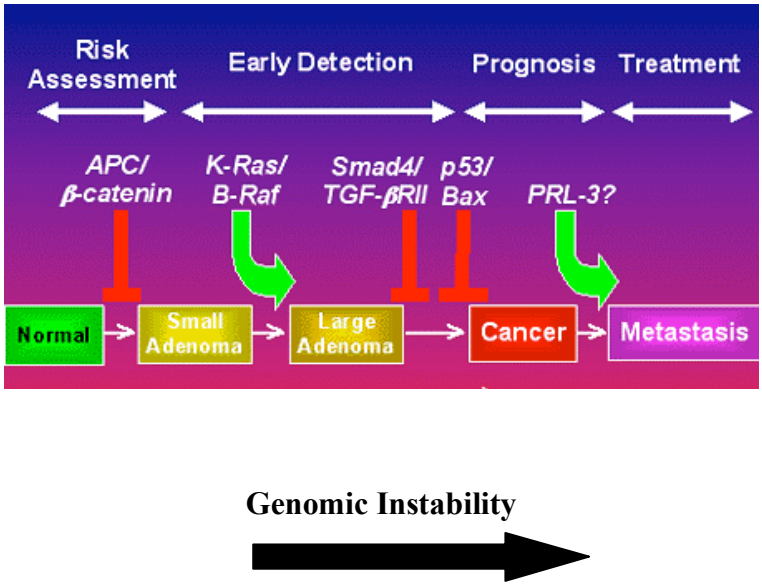


Figure 2.4: Stages and mutations associated with colorectal cancer. Initial transformation of normal colonic mucosa is associated with loss of adenomatous polyposis coli (APC)/β-catenin interaction. Progression to small and large colonic adenomas is associated with genomic instability and the accumulation of further genetic mutations including activation of the *ras* oncogene and loss of p53. Adapted from Kinzler and Vogelstein (1996).

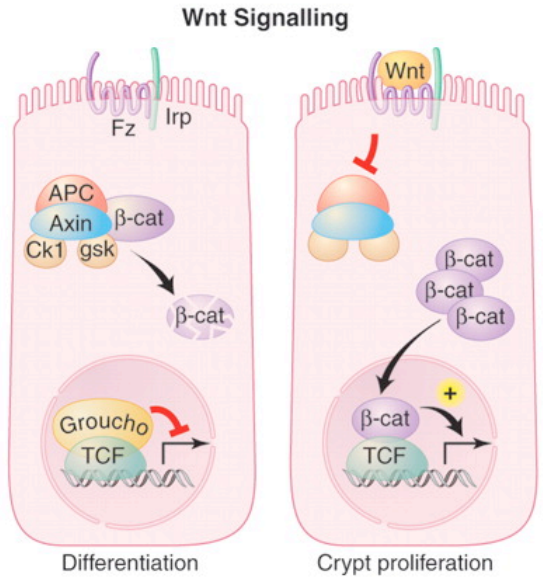


Figure 2.5: Interaction between APC and β-catenin in the Wnt signaling pathway. In the absence of Wnt binding to its frizzled receptor (Fz), β-catenin is found in complex with APC and E-cadherin to mediate cell-cell contacts (not shown). Free β-catenin in the cytosol is targeted for ubiquitination by APC in complex with other cellular factors. Upon Wnt signalling, β-catenin ubiquitination is blocked and it translocates to the nucleus where it binds TCF transcription factors and activates the transcription of genes. From Radtke and Clevers (2005).

cases of sporadic tumors that retain functional APC often have mutated β-catenin with an elevated activity (Molatore and Ranzani, 2004). Other significant alterations observed in colorectal carcinogenesis include allelic losses of tumor suppressor genes. For example, loss of a portion of chromosome 17p which contains the gene for the tumor suppressor p53, is observed in more than 75 % of colorectal carcinomas (Vogelstein *et al.,* 1988). In addition, loss of the tumor suppressor gene deleted in colorectal cancer (DCC) is observed in more than half of colorectal cancers when regions of chromosome 18q are lost (Vogelstein *et al.,* 1988). The normal DCC gene product plays a role in the induction of apoptosis. Upon loss of active DCC gene product, cells become resistant to apoptosis resulting in increased resistance to chemotherapeutic agents (Rivka *et al.*, 2004). In addition to tumor suppressor gene inactivation, down regulation of mismatch repair (MMR) genes has been observed in 10-15 % of sporadic colorectal carcinomas. The loss of these genes including MLH1 or MSH2 results in the expansion or deletion of repetitive microsatellite sequences throughout the genome having a deleterious effect on the expression of a number of gene products (Ionov *et al.,* 1993).

In addition to genetic mutations, epigenetic modifications of genomic DNA are frequently observed in colorectal carcinomas (reviewed in Kondo and Issa, 2004). Increased levels of DNA methylation have been observed in colorectal carcinomas compared to normal colonic mucosa. This hypermethylator phenotype is referred to as CpG island methylator phenotype (CIMP) and is observed in 50 % of sporadic colorectal carcimonas (Grady, 2004). In addition to DNA methylation, histone modifications including hypoacetylation and methylation of lysine 9 on histone 3 have been shown to contribute to tumor suppressor gene inactivation in colorectal carcinomas (reviewed in Kondo and Issa, 2004).

Although the majority of colorectal cancer cases are sporadic in nature, 15 % of colorectal cancer cases are hereditary where mutations exist in germline cells and are passed on from one generation to the next (Kinzler and Vogelstein, 1996). The two main types of hereditary colorectal cancer are familial adenomatous polyposis coli (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). FAP is an autosomal

dominantly inherited disease that affects approximately 1 in 7000 individuals (Bodmer *et al*., 1987; Solomon *et al*., 1987). These individuals have inherited a mutated copy of the APC gene which codes for a truncated protein. Following the accumulation of further mutations including inactivation of the remaining APC allele, these individuals in their teens and young adulthood develop hundreds to thousands of adenomatous polyps that are benign, but have the potential to progress to a more invasive stage of carcinogenesis if the colon is not removed (Kinzler and Vogelstein, 1996). HNPCC is also an autosomal dominant disorder, however it arises from the inheritance of mutations of the mismatch repair genes (MMR) resulting in allelic losses of microsatellite regions and microsatellite instability (Lynch *et al*., 1985). Hallmarks of this disease include the presence of up to 100 colonic polyps with the average age of onset of colorectal carcinoma occurring at 40 years of age (Wang *et al*., 2006).

2.2.2 Stages of tumorigenesis

An immense amount of research has focused on identifying the genetic and morphological changes associated with the process of colorectal carcinogenesis, beginning with the initial transformation of normal colonic epithelial cells (Figure 2.6) (Fearon and Vogelstein, 1990). The first observable indication of colorectal neoplasia is the development of pre-neoplastic lesions called aberrant crypt foci (ACF) which arise from hyperproliferating colonic epithelial cells and consist of cells of either normal or dysplastic morphology (Molatore and Ranzani, 2004). Although not all ACF progress to colorectal cancer, studies have shown that ACF, which are dysplastic in nature, are precursors of colorectal cancer (Cheng and Lai, 2003). Microscopically, ACF are characterized by the clustering of aberrant crypts that are distinguishable from normal colonic crypts by having a thicker epithelial lining (Figure 2.7) (Bird and Good, 2000; Cheng and Lai, 2003). Analysis of genetic alterations in these early ACF lesions revealed elevated levels of *ras* oncogene product as well as mutations in APC and p53 genes (Vogelstein *et al.,* 1988).

Previous work by Sugarbaker *et al.* (1985) revealed that most colorectal carcinomas arise from adenomas that progressively increase in size, dysplasia and

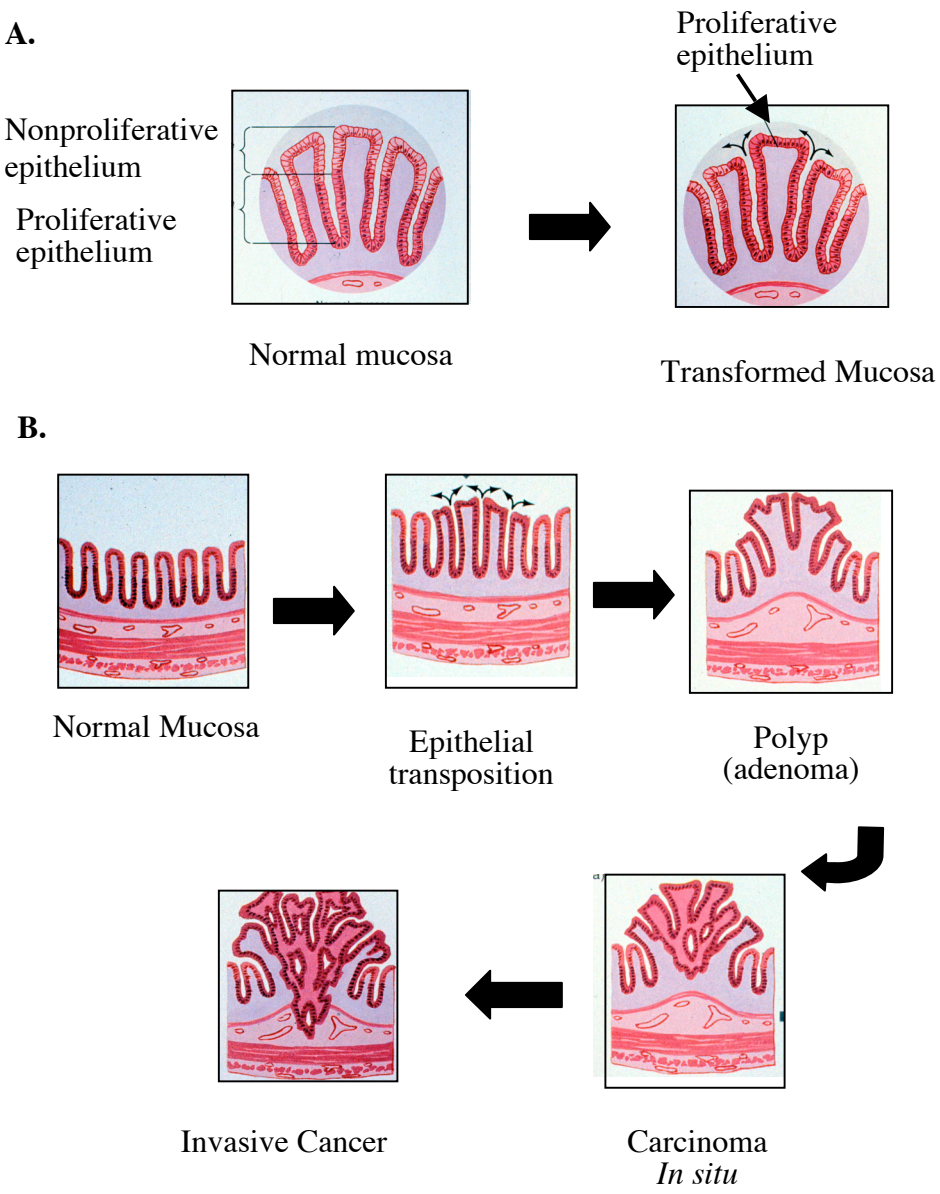


Figure 2.6: Transformation of normal mucosa and colon cancer progression. Panel A indicates the difference between the proliferative epithelium in normal mucosa as compared to transformed colonic mucosa. Panel B depicts the morphological changes that result during colon cancer progression.

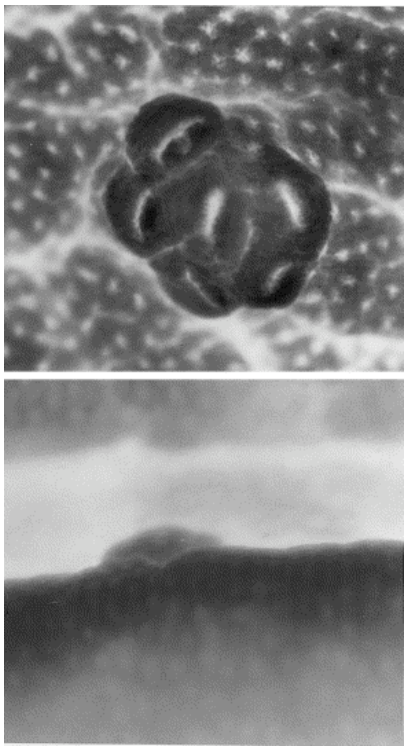


Figure 2.7: Visualization of aberrant crypt foci (ACF) in fixed colon. Stained ACF show crypts with thicker epithelial lining. From Bird and Good (2000).

villous morphology. After development of dysplastic ACF, further genetic and epigenetic events lead to the development of an early adenoma (Figure 2.4). Early adenomas are defined as those equal to or less than 1.0 cm in size. Progression to intermediate stage adenomas that are greater than 1.0 cm in diameter requires enhanced cellular proliferation, which is often accounted for by activation of the *ras* oncogene. Chromosomal losses, including loss of 18q which contains genes encoding the tumor suppressor gene deleted in colon cancer (DCC) as well as SMAD2 and SMAD4 genes can lead to progression to late stage adenomas, characterized by a size greater than 1.0 cm and acquisition of a villous morphology (Vogelstein *et al.,* 1988). Further chromosomal loss, including loss of the p53 gene on chromosome 17p, has been associated with the transition from adenoma to carcinoma characterized by local invasion and the potential for metastasis (Fearon and Vogelstein, 1990). Although genetic and epigenetic alterations have been associated with discreet changes in morphology (Figure 2.6) and stages of colorectal carcinogenesis, it was postulated that it is the accumulation of mutations, rather than the order in which they occur that is most significant to the development of colorectal cancer (Figure 2.4) (Fearon and Vogelstein, 1990).

2.2.3 Invasion and metastasis

Local invasion of malignant colon carcinoma into the surrounding stromal tissue is followed by intravasion into lymphatic and blood vessels and the establishment of a tumor at a secondary site. At the time of diagnosis it has been documented that 30-50% of patients with colorectal carcinoma exhibit metastasis in regional lymph nodes and 10-30% of patients exhibit hepatic metastases (Jemal *et al.,* 2005). Although liver is the main site of metastasis in patients with colorectal carcinoma, metastatic disease can be present in other organs including the lung, brain and bone marrow (reviewed in Zucker and Varcirca, 2004).

In order for metastasis to occur, cells with metastatic potential must escape from the primary tumor by reducing cell-cell contacts with neighbouring cells as well as reduce cell-ECM contacts. If metastasis is to occur, cells must attain a more motile phenotype. In colorectal cancer, it has been observed that invasion by carcinomas

involves a loss of epithelial cell characteristics and a gain of mesenchymal cell characteristics (Brabletz *et al*., 2005). This process, referred to as the epithelial-mesenchymal transition (EMT), involves a dedifferentiation process that allows cells to become invasive. In fact, Jass *et al*. (2003) have shown that dedifferentiation of colorectal carcinomas correlates with poor clinical prognosis. Dedifferentiation involves the activation of the Wnt pathway (Figure 2.5). When the Wnt pathway is not stimulated, β-catenin is found in the cytoplasm of cells complexed with the cytoplasmic domain of E-cadherin cell surface receptors. This complex is necessary for the maintenance of cell-cell contacts between epithelial cells in normal tissues. The inactivation of the tumor suppressor APC in colorectal cancer results in the accumulation of cytosolic β-catenin which is translocated to the nucleus, resulting in enhanced transcription of a number of genes such as *Id2* (Rockman *et al.,* 2001) and *Cdx-1* (Lickert *et al*., 2000) that have been implicated in the EMT process. Loss of cytosolic β-catenin also results in reduced binding to E-cadherin, resulting in decreased cell-cell contacts. Previous studies have shown that Wnt signaling as well as the nuclear accumulation of β-catenin can result in the dedifferentiation of epithelial cells to mesenchymal cells during embryogenesis (Logan *et al.,* 1999). This pathway likely plays a role in the dedifferentiation of colorectal carcinoma cells during invasion. After invasion of carcinoma cells, redifferention occurs resulting in metastatic tumor cells at a secondary site assuming once again an epithelial phenotype (Brabletz *et al.,* 2005).

Once carcinoma cells have acquired a motile phenotype they must be able to invade into the surrounding stromal tissue. There are a number of proteinases that are involved in the degradation of the extracellular matrix during the invasion of colorectal carcinomas. For example, overexpression of the serine proteinase trypsin results in enhanced digestion of type I collagen in the ECM in colorectal carcinomas and is associated with poor prognosis and shorter disease-free survival (Soreide *et al.,* 2006). In addition to the cleavage of type I collagen, trypsin is responsible for activating matrix metalloproteinases MMP-2, -7 and -9 resulting in enhanced degradation of the extracellular matrix. In fact, the co-expression of trypsin with MMP-2, -7 and -9 in colorectal carcinomas has been implicated in enhanced cellular proliferation, progression and invasion (Soreide *et al.,* 2006).

Previous studies have revealed that MMPs are the main proteinases involved in the invasion of colorectal carcinomas. Specific MMPs that have been implicated in colorectal cancer include MMP-1, -2, -3, -7, -9, -12, -13 and MT1-MMP (reviewed in Zucker and Vacirca, 2004). Although MMPs play a role in colorectal cancer, MMP-7 appears to have the most significant effect not only on colorectal tumorigenesis, but also in invasion and metastasis. The expression of MMP-7 is observed at early stages of tumor progression and has been implicated in enhanced cellular proliferation as well as the progression from adenoma to invasive carcinoma. This MMP-7 expression was observed to be localized in the lumen of dysplastic colon tumors isolated from mice (Wilson *et al.,* 1997). Studies have revealed that MMP-7 is expressed by 90% of colonic adenocarcinomas and its expression is correlated with the presence of lymph node as well as distant metastases (Newell *et al*., 1994). One possible explanation for the enhanced expression of MMP-7 is that its gene is a target of transcription factors activated by nuclear accumulation of β-catenin, which occurs when functional APC is absent (Aberle *et al*., 1997).

As previously mentioned, MMPs are not only involved in the degradation of the ECM, but they are also responsible for the cleavage of non-ECM proteins resulting in an altered function of the cleaved protein. For example, cleavage of Fas ligand (FasL) from the cell surface by MMP-7 results in release of soluble FasL from the cell surface and altered cell survival (Powell *et al*., 1999). In addition, MMP-7 and MMP-3 cleave osteopontin, a secreted glycoprotein that interacts with cell surface receptors such as integrins and CD44 resulting in enhanced migration of cells towards cleaved osteopontin (Agnihotri *et al.,* 2001).

The activity of MMPs is tightly regulated by inhibitors of MMP activity, which include tissue inhibitors of matrix metalloproteinases (TIMPs) as well as α_2-macroglobulin. There are four TIMPs (1-4) that are responsible for inhibiting the activity of various MMP activities. The ratio of TIMP: MMP is important for the regulation of MMP activity. For example, when TIMP levels are elevated, MMP activity is suppressed and when TIMP levels are reduced, the MMP activity is elevated (reviewed in Zucker and Vacirca, 2004). Specifically, TIMP-2 binding to proMMP-2 aids in the formation of a complex with membrane-bound MT1-MMP, thereby

enhancing the activation of MMP-2 (Sato *et al*., 1994). Another inhibitor of soluble MMPs is the plasma protein α_2-macroglobulin. Alpha-macroglobulins form complexes with a wide range of proteinases effectively reducing their proteolytic activities (Sottrup-Jensen and Birkedal-Hansen, 1989).

Once tumor cells have accessed lymphatic or blood vessels, this usually leads to the establishment of hepatic metastases in patients with colorectal cancer (Rudmik and Magliocco, 2005). Embolization of tumor cells in the vasculature allows for enhanced cell survival by reducing detection by the immune system. The expression of cancer cell surface integrins plays a role in mediating tumor cell arrest in hepatic vessels, allowing the development of hepatic metastases (Rudmik and Magliocco, 2005). Another colorectal carcinoma cell surface protein involved in the establishment of hepatic metastasis is carcinoembryonic antigen (CEA), which binds to specific receptors present on hepatic cells. This interaction stimulates the secretion of cytokines that act to elevate the expression of intercellular adhesion molecule-1 (ICAM-1) on the hepatic endothelium, which enhances the binding of colorectal carcinoma cells (Wagner *et al*., 1992).

2.2.4 Detection and treatment

The establishment of metastatic disease accounts for the majority of cancer-related deaths (Weigelt *et al*., 2005). Thus, early detection of cancer before metastasis has occurred is critical to decreasing cancer mortality rates. In the case of colorectal carcinoma, screening of the colon for potential lesions does not usually occur until after the age of 50, unless there is a history of hereditary colorectal cancer. Common screening procedures for colorectal cancer include the fecal occult-blood test, sigmoidoscopy, colonoscopy and double-contrast barium enema (reviewed in Kahi and Rex, 2004). The fecal occult-blood test (FOBT) is the most common type of screening procedure performed in the United States. FOBT involves examining feces for the presence of blood, which may indicate the presence of a colorectal polyp. A positive result would be followed by a sigmoidoscopy or colonoscopy. One disadvantage of this screening procedure is that colorectal polyps can bleed intermittently, so the inability to detect blood in the feces does not conclusively indicate a colon free of lesions.

Another screening procedure is sigmoidoscopy, which examines the distal colon. This procedure is easier to perform than a colonoscopy as it does not require that the patient be sedated, however it is less sensitive and is not able to detect potential lesions in the proximal colon (reviewed in Kahi and Rex, 2004). The double-contrast barium enema (DCBE) is a screening procedure involving the radiologic examination of the entire colon. The use of this procedure is on the decline because it is not as sensitive as colonoscopy in detecting colorectal lesions and it is uncomfortable for patients (reviewed in Kahi and Rex, 2004).

Colonoscopy is currently the most sensitive procedure for screening for the presence of colorectal lesions. This procedure is able to scan the whole colon and is used in combination with a polypectomy to remove any potential polyps observed at the time the colonoscopy is performed. Some disadvantages of this procedure is that it requires extensive preparation of the bowels which can be cumbersome for the patient and it also requires sedation of the patient (reviewed in Kahi and Rex, 2004).

Some emerging techniques in the detection of colorectal lesions involve fecal DNA tests. These assays involve examination of feces for the presence of DNA containing mutations in oncogenes and tumor suppressor genes that would be shed by colorectal neoplasms. A multi-target assay is currently being tested that examines feces for the presence of DNA mutations in APC, K-ras, and p53 which account for all cases of FAP and approximately 85% of sporadic colorectal cancers. Advantages of this screening procedure are that it is sensitive and non-invasive. Unfortunately, at the moment this screening procedure is quite expensive and not as sensitive as a colonoscopy (reviewed in Kahi and Rex, 2004). Analysis of serum markers for colorectal cancer revealed carcinoembryonic antigen (CEA) as well as carbohydrate antigen 19-9 (CA19-9) serum levels were elevated in patients with colorectal cancer (Srivastava *et al*., 2001). Following immunohistochemical analysis of colorectal tumors, it was revealed that the increased expression of CEA by colorectal carcinomas correlated with poor prognosis and disease recurrence making CEA the only reliable serum marker to date for determining the prognosis of colorectal cancer (Hilska *et al*., 2005).

Once a colorectal polyp is detected and removed via polypectomy, histological analysis is performed to determine the stage of progression, course of treatment and prognosis. Initially, colorectal cancer was staged according to Duke's staging, which was further modified by Astler and Coller (Figure 2.8). In this staging system, tumors are classified as either Duke's A, B1, B2, C1, C2 or D. Duke's A tumors are restricted to the mucosa. Duke's B1 tumors display invasion through to the muscularis propria while B2 tumors invade through the serosa. Duke's C1 and C2 tumors display invasion similar to B1 and B2 tumors, respectively, but also include lymph node involvement. Finally Duke's D tumors are those associated with the presence of distant metastases (Astler and Coller, 1954).

A more current method of classification system has been implemented and is referred to as the TNM (tumor, node, metastasis) classification system where T refers to the stage of the tumor. In this classification system, Duke's A, B1, B2 are replaced by T1, T2 and T3 respectively. In addition, Duke's C tumors are described by N, which refers to the number of lymph nodes that are involved with N1 corresponding to 1-3 lymph nodes and N2 corresponding to more than 3 lymph nodes. Duke's D tumors with the presence of distant metastasis are referred to as M1 tumors in this classification system.

Cancer is often classified according to stages, which dictate future treatment options and prognosis. In colorectal cancer, stage I disease indicates the presence of T1 or T2 tumors. Stage II disease refers to tumors described as T3. Stage III disease involves the presence of metastatic disease in the lymph nodes and stage IV disease involves the presence of distant metastases to the liver or other organs (Figure 2.8). Patients with stage I colorectal cancer exhibit no signs of invasive disease and removal of the polyp or tumor completes the course of treatment. In contrast, patients with stage II, III or IV disease require removal of the primary tumor followed by adjuvant therapy in an attempt to eliminate metastases. The presence of local invasion of the primary tumor in patients with advanced stages of colorectal cancer requires that entire sections of the colon be removed in order to eliminate as much of the invaded tissue as possible. Following surgical resection of the colon, patients are commonly treated with the

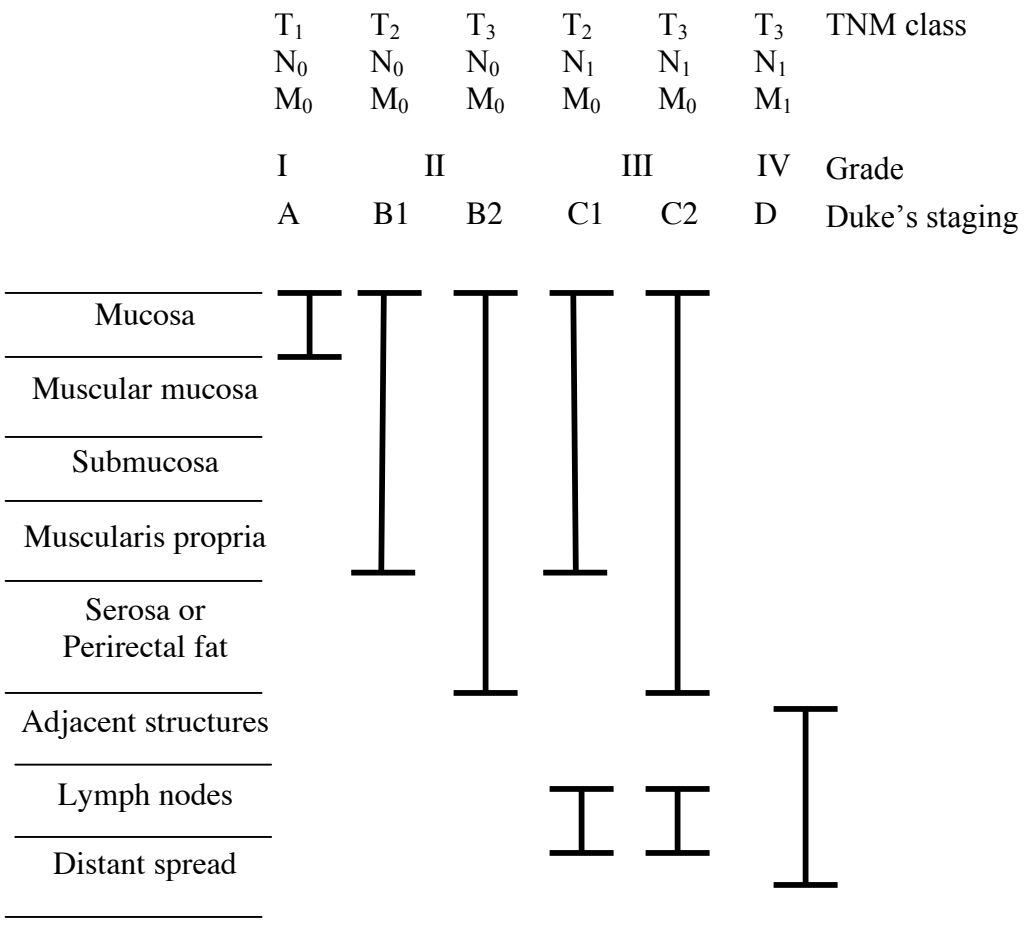


Figure 2.8: Astler-Coller modified Duke's and TNM staging for colorectal cancer.

chemotherapeutic agents 5-fluorouracil and leucovorin that inhibit DNA synthesis in an attempt to eliminate metastases (reviewed in Xiong and Ajani, 2004).

2.3 ROLE OF GLYCOSYLATION IN CANCER AND METASTASIS

In order to reduce the incidence of death associated with cancer, it will be necessary to develop improved methods for the detection, prevention and treatment of cancer. One approach could involve the identification of novel biomarkers as well as cancer cell-specific alterations. The aberrant expression of oncogenes and tumor suppressor genes occur in all types of cancer and collectively these alterations result in the generation of transformed cancer cells with altered characteristics (reviewed in Hanahan and Weinberg, 2000). Cell surface carbohydrates are differentially expressed during normal processes such as embryogenesis and cellular differentiation, however, cellular transformation is also accompanied by altered expression of cell surface glycans which results in enhanced motility and malignant potential (Dennis, 1992; Saitoh *et al*., 1992). In fact, one of the most consistent biochemical changes associated with colon tumorigenesis and metastasis is altered expression and/or structure of cell-associated complex carbohydrates (Dennis *et al.,* 1987; Fernandes *et al.,* 1991). Moreover, cancer progression is directly associated with increased levels of aberrant oligosaccharides which are believed to contribute to tumor cell motility and invasion by altering cell-cell and cell-extracellular matrix interactions (Kannagi 1997; Ochwat *et al*., 2004).

2.3.1 Types of cell-surface carbohydrates

Carbohydrates are generally linked to macromolecules to generate glycoproteins and glycosphingolipids. The glycosylation of proteins is important in mediating proper folding, subcellular localization, rate of degradation and function (Opdenaker *et al.,* 1993). Glycoproteins are glycosylated in the ER and Golgi apparatus and are usually secreted extracellularly or embedded into membranes, including the plasma membrane. The plasma membrane of the cell is composed of glycosphingolipids where the extent of glycosylation of the lipids can affect membrane rigidity and functions of membrane receptors (Chammas *et al*., 1991). Cell-surface carbohydrates on both

glycosphingolipids and glycoproteins have been shown to be important in mediating cell-cell and cell-ECM interactions (Kornfeld, 1980).

2.3.2 Changes in cell surface carbohydrates

2.3.2.1 Alterations to glycosphingolipids

The lipid bilayer of the plasma membrane is composed of glycosphingolipids, which are composed of ceramide molecules modified by core carbohydrate structures. Glycosphingolipids are classified as either ganglio-series, globo-series, lacto-series type I or lacto-series type II based on the core structure that is present (Figure 2.9) (Hakomori, 2003). The carbohydrate structures present on cell surface glycosphingolipids serve as ligands for lectins such as selectins, which play a role in mediating cellular adhesion (Fukada 1996; Fukada *et al*., 1999). In addition, carbohydrate structures also have the potential to form interactions with other carbohydrates mediating cell-cell interactions as well as cellular adhesion (Hakomori, 2003). Further modification of glycosphingolipids by negatively charged sialic acid residues results in the generation of acidic gangliosides. Studies have revealed that glycosphingolipids and specifically gangliosides in the lipid bilayer can affect the activity of cell surface receptors including growth factor receptors and integrins resulting in altered cell growth and motility (Weis and Davis, 1990). The elevated expression of aberrant carbohydrate structures including poly-N-acetyllactosamine [i.e. repeating type 2 (Galβ1-4GlcNAcβ-R) chains] and Lewis antigens (Figure 2.10) modifying glycosphingolipids have been attributed to the promotion of invasive cells and enhanced metastasis with reduced survival in many types of cancer (Hakomori, 2002). In addition to elevated tumor-associated carbohydrate structures, the increased production of ganglioside GD3 by cancer cells promotes tumor motility and growth by enhancing angiogenesis through increased expression of VEGF (Zeng *et al*., 2000).

2.3.2.2 Alterations to glycoproteins

Glycoproteins consist of carbohydrate moieties attached to a polypeptide backbone with two main types of glycosidic linkages observed. O-linked oligosaccharides result from an O-glycosidic bond between the hydroxyl group of

A. **Lacto-series type 1: Lc_4** Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-Cer

B. Lacto-series type 2: nLc_4 Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer

nLc_6 Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3
Galβ1-4Glcβ1-Cer

C. Globo-series: **Gb_3** Galα1-4Galβ1-4Glcβ1-Cer

Gb_4 GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer

Gb_5 Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer

D. Ganglio-series: **Gg_3** GalNAcβ1-4Galβ1-4Glcβ1-Cer

Gg_4 Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer

Figure 2.9: Core carbohydrate structures of glycosphingolipids.
Carbohydrate structures consisting of galactose (Gal), N-acetylglucosamine (GlcNAc), glucose (Glc), N-acetylgalactosamine (GalNAc) monosaccharides are linked to ceramide (Cer) molecules. From Hakomori, 2003.

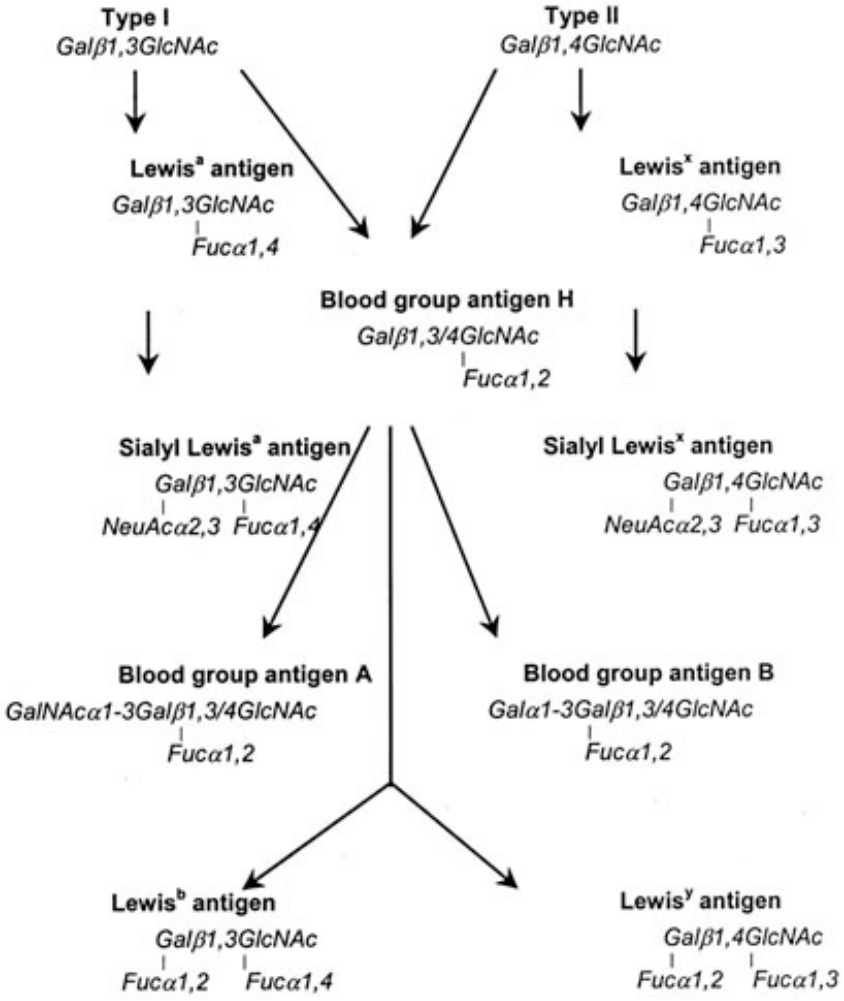


Figure 2.10: Schematic of the biochemical pathways leading to the production of Lewis and histo-blood group antigen from precursor antigens. From Hittelet *et al.* (2003).

serine or theonine residues in the polypeptide backbone and the sugar structure. On the other hand, carbohydrate moieties which form an N-glycosidic bond with the amide group of asparagine residues in the polypeptide backbone, are referred to as N-linked oligosaccharides. The expression of glycoproteins bearing elevated levels of cancer-associated carbohydrates or carbohydrates with altered structures, is a common alteration in cancer (Ogata *et al*., 1976; Dennis *et al*., 1987; Fernandes *et al*., 1991).

2.3.2.2.1 Biosynthesis of O-linked oligosaccharides

O-linked glycans have been implicated in a number of processes including cell adhesion, protecting epithelial surfaces, blood clotting and development during embryogenesis (Varki, 1993; Brockhausen and Kuhns, 1997). The addition of O-linked oligosaccharides occurs post-translationally in the Golgi apparatus and involves glycosyltranferases, sulfotransferases and nucleotide sugar donors. Initially an N-acetylgalactosamine (GalNAc) residue is joined through an O-glycosidic linkage to the hydroxyl group of a serine or threonine residue by the enzyme α–GalNAc-transferase. This structure, referred to as the Tn antigen, is elongated to generate one of eight different core structures that have been identified in mammalian glycoproteins (Figure 2.11) (reviewed in Brockhausen, 1999). Once the O-glycan core has been generated, it can be further elongated by the addition of poly-N-acetyllactosamine repeating units (Ujita *et al*., 2000), which can be terminated with either sialic acid residues (Kuhns et al., 1993), histo-blood group ABH antigens (Hitoshi *et al*., 1996) or Lewis antigens (Kufowska-Latallo *et al*., 1990) (Figure 2.10)**.** O-glycan core glycosylation can also be terminated by addition of sialic acid residues, which prevents further elongation of carbohydrate chains. Finally, GlcNAc or Gal residues present in O-glycans can be sulfated by various sulfotransferases (Capon *et al*., 1997). The over-expression of O-linked oligosaccharides, including sialyl-Tn (STn) has been observed in a number of different types of cancer (Itzkowitz *et al*., 1990; Kakeji *et al*., 1995). Recently, Biomira Inc. (Edmonton, AB, Canada) developed a therapeutic cancer vaccine by targeting cancer cells which express enhanced STn antigen. This vaccine has been shown to enhance the immune response to STn antigen in patients suffering from breast, ovarian

Core 1 Galβ1-3GalNAc-

Core 2 GlcNAc
|β1-6
Galβ1-3GalNAc-

Core 3 GlcNAcβ1-3GalNAc-

Core 4 GlcNAc
|β1-6
GlcNAcβ1-3GalNAc-

Core 5 GalNAcα1-3GalNAc-

Core 6 GlcNAcβ1-6GalNAc-

Core 7 GalNAcα1-6GalNAc-

Core 8 Galα1-3GalNAc-

Figure 2.11: O-glycan core structures. Adapted from Brockhausen (1999).

and colon cancer resulting in enhanced survival compared with control patients. This vaccine is currently in phase III clinical trials (Biomira Inc., 2003).

2.3.2.2.2 Biosynthesis of N-linked oligosaccharides

In contrast to O-linked oligosaccharides, N-linked oligosaccharides are added to proteins co-translationally in the ER by the oligosaccharidyl transferase complex, which transfers a pre-assembled 14-sugar oligosaccharide moiety from its membrane-anchored dolichol-phosphate linked precursor ($Glc_3Man_9GlcNAc_2$-P-P-Dol) to accessible asparagine residues located within the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline (Figure 2.12) (Gavel and von Heijne, 1990; reviewed in Kelleher and Gilmore 2006). Synthesis of the 14-sugar oligosaccharide precursor begins in the cytosol by the transfer of GlcNAc and subsequently Man from the nucleotide sugar donors GDP-GlcNAc and GDP-Man, respectively, to membrane anchored dolichol-phosphate by cytosolic N-acetylglucosaminyl transferases and mannosyl transferases, respectively. The resulting $Man_5GlcNAc_2$-P-P-Dol intermediate is flipped across the ER membrane where lumenal glycosyltransferases modify the oligosaccharides with additional mannose and glucose residues (Varki *et al.,* 1999; Helenius and Aebi, 2002).

If the protein is properly folded, the terminal glucose residues and one of the α1,2-linked mannose residues are removed by glucosidases I and II and α-mannosidase I, respectively. Glycoproteins modified with the resulting high-mannose type oligosaccharides (Figure 2.12 and Figure 2.13, panel A) are transported to the cis-Golgi where they are modified by additional glycosidases and glycosyltransferases (reviewed in Helenius and Aebi, 2004). This includes removal of the remaining α1,2-linked mannose residues by Golgi α-mannosidase I and addition of a GlcNAc residue by N-acetylglucosaminyltransferase I (GnT-I) resulting in the formation of hybrid-type oligosaccharides (Figure 2.12 and Figure 2.13, panel B) (Kornfeld and Kornfeld 1985). Removal of α-3 and α-6-linked mannose residues by Golgi α-mannosidase II and subsequent addition of GlcNAc residues by N-acetylglucosaminyltransferases result in the formation of complex-type oligosaccharide structures (Figure 2.13, panel C). These oligosaccharides can be further modified by addition of polylactosamine structures,

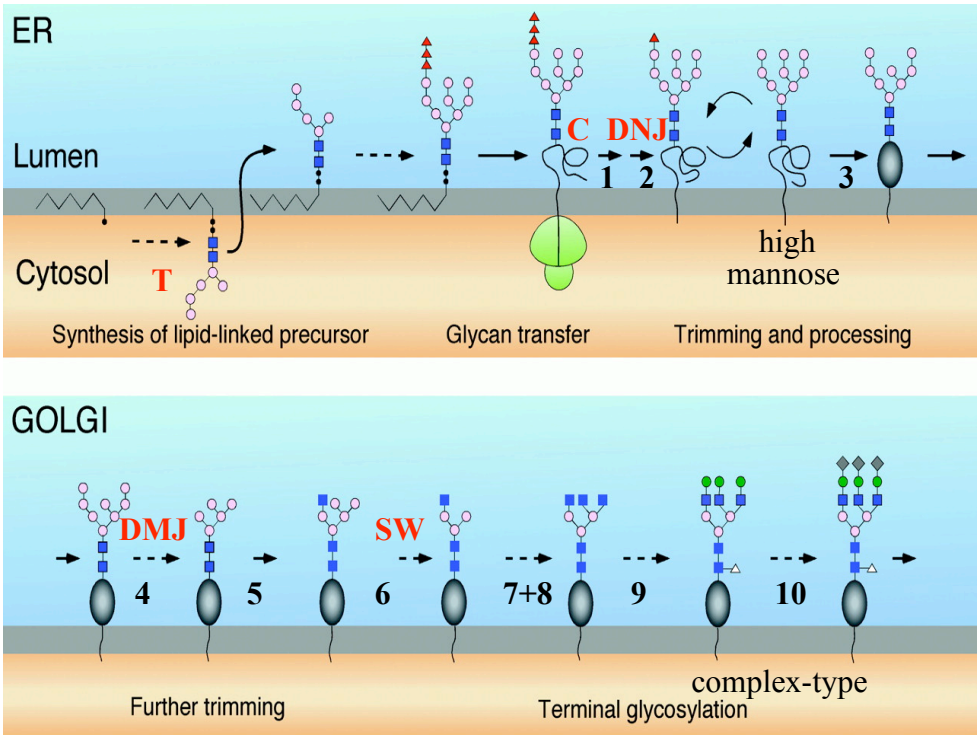


Figure 2.12: Biosynthesis of N-linked oligosaccharides. Schematic representation of N-linked biosynthesis where blue squares correspond to GlcNAc residues, white circles represent mannose residues, red triangles represent glucose residues, green circles are galactose residues and grey diamonds represent terminal structures such as sialic acid or fucose residues. Enzymes are numbered as 1 (ER α-glucosidase I), 2 (ER α-glucosidase II), 3 (ER α-mannosidase), 4 (Golgi α-mannosidase I), 5 (N-acetylglucosaminyltransferase I), 6 (Golgi α-mannosidase II), 7 (N-acetylglucosaminyl transferase II), 8(N-acetylglucosaminyltransferase III), 9 (galactosyltransferases) and 10 (sialyltransferases or fucosyltransferases). Inhibitors of carbohydrate processing are indicated: T, tunicamycin; C, castanospermine; DNJ, 1-deoxynojirimycin; DMJ, 1-deoxymannojirimycin and SW, swainsonine. Adapted from Helenius and Aebi (2001).

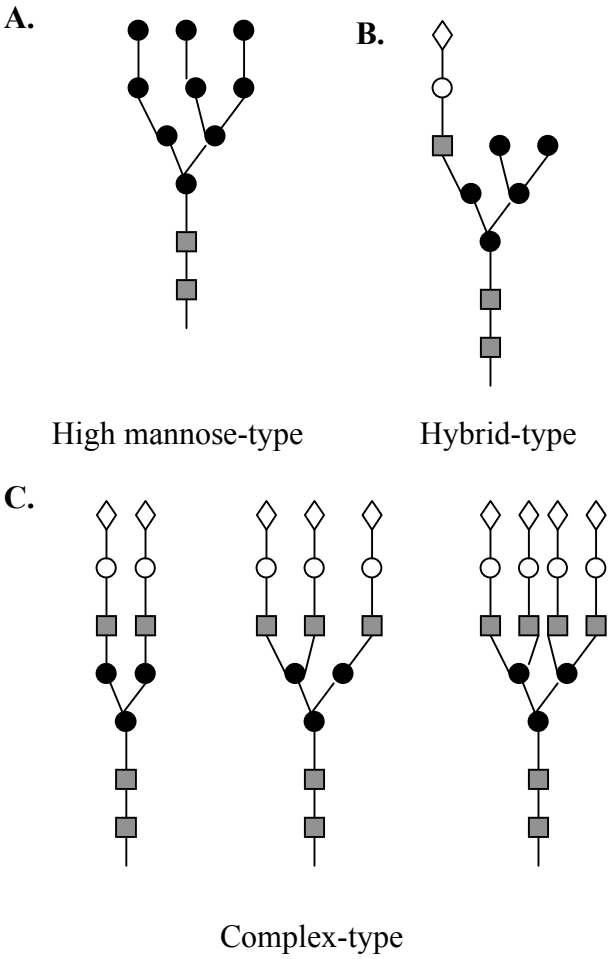
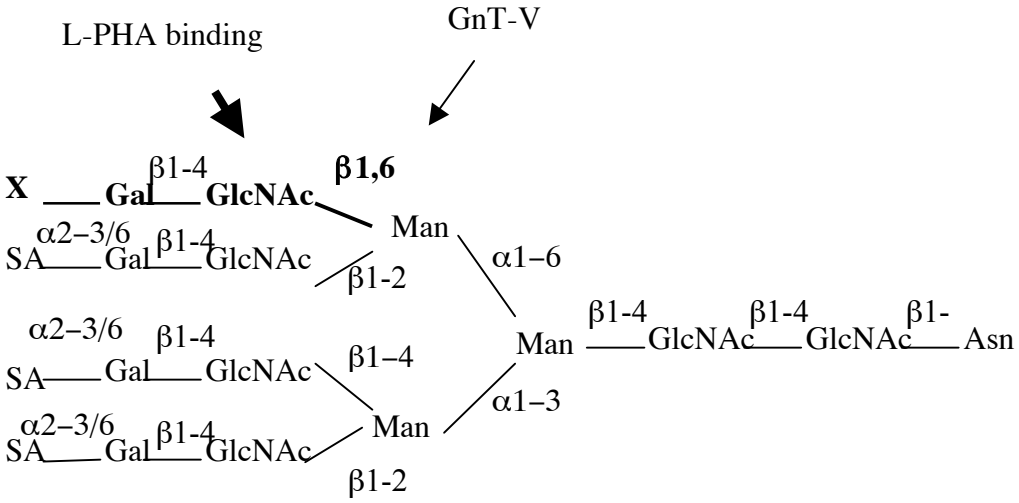


Figure 2.13: Examples of branching structures observed in N-linked oligosaccharides. Monosaccharides are represented as: GlcNAc, grey squares; Man, black circles; Gal, white circles; sialic acid, white diamonds.



X = sialic acid (SA), polylactosamine (i.e.Galβ1-4GlcNAc)$_n$, Le^x

Figure 2.14: β1-6 branched N-linked oligosaccharides.

Lewis antigens and sialic acid residues (Figure 2.10, Figure 2.12 and Figure 2.13, panel C) (reviewed in Dennis *et al*., 1999a ; reviewed in Jones *et al.,* 2005).

2.3.2.2.2.1 Altered expression of β1-6 branched N-linked oligosaccharides in cancer

One of the earliest changes associated with cellular transformation is the presence of increased levels of highly branched complex-type N-linked oligosaccharides resulting in the production of glycoproteins with elevated molecular weights (Ogata *et al*., 1976). Upon further examination, it was revealed that elevated levels of these complex-type N-linked oligosaccharides were associated with increased levels of the Golgi enzyme N-acetylglucosaminyl transferase V (GnT-V), also known as mannoside acetyl glucosaminyl transferase 5 (Mgat5) (Cummings and Kornfeld, 1982). This enzyme initiates the synthesis of β1-6 branching of N-linked oligosaccharides (Figure 2.14) (reviewed in Pierce *et al*., 1997). The extent of β1-6 branching of N-linked oligosaccharides expressed by cancer cells was determined by employing the plant lectin phytohemagglutinin-leukoagglutinin (L-PHA) from *Phaseolus vulgaris* which binds to the Galβ1,4GlcNAcβ1,6 (Galβ1,4GlcNAcβ1,2)Manα portion of tri- and tetraantennary N-glycans formed by GnT-V (Cummings and Kornfeld, 1982). The increased expression of β1-6 branched N-linked oligosaccharides on the cell surface was observed following transformation of cells with an oncogenic virus such as polyoma virus or following transfection with the *ras* oncogene (Yamashita *et al.,* 1985; Dennis *et al.,* 1987,1989). In addition to being present on the surface of cells transformed *in vitro*, elevated amounts of β1-6 branched N-linked oligosaccharides were also observed in a number of human cancers, including melanoma as well as breast and colon cancer (Fernandes *et al*., 1991). In fact, the amount L-PHA staining detected in human colorectal cancer sections correlated with tumor recurrence, patient survival, and lymph node metastasis (Fernandes *et al*., 1991; Seelentag et al., 1998). Elevated levels of β1-6 branched N-linked oligosaccharides are believed to contribute to tumor cell motility and invasion by altering cell-cell and cell-extracellular matrix interactions (Saitoh *et al*., 1992 ; Zheng *et al*., 1994).

Further evidence in support of the contribution of β1-6 branched N-linked oligosaccharides to increased metastatic potential of cancer cells is their role in mediating the loss of cell-cell and cell-ECM contacts. For example, the presence of increased β1-6 branched N-linked oligosaccharides on CD44, the cell surface receptor for hyaluronic acid, reduces the affinity of the receptor for its substrate, effectively reducing the contact of the cell surface with the ECM (Schmits *et al*., 1997). Similarly, cells transfected with GnT-V exhibited reduced binding to ECM proteins which was associated with increased expression of β1-6 branched oligosaccharides on integrin subunits β1, α5 and αv (Demetriou *et al*., 1995; Guo *et al*., 2002). Loss of cell-cell contacts has also been observed with increased expression of these oligosaccharides on the extracellular domain of E-cadherin which reduces the binding to E-cadherins on the surface of adjacent cells, resulting in enhanced cell motility (Birchmeier, 1995).

Consistent with increased levels of β1-6 branched N-linked oligosaccharides in cancer cells, elevated expression of GnT-V was observed in a number of cancers where it was associated with increased metastatic potential (Seberger and Chaney 1999; Murata *et al*., 2000). For example, the extent of metastasis observed in human colorectal cancer tumors correlated with the amount of GnT-V expressed by cancer cells (Murata *et al*., 2000). In addition, non-tumorigenic cells that were transfected with GnT-V displayed characteristics of transformed cells, including loss of contact inhibition and enhanced motility in tissue culture. Furthermore, in experimental metastasis studies, injection of GnT-V-transfected mammary carcinoma cell lines into nude mice resulted in enhanced establishment of lung metastases compared to non-transfected controls (Seberger and Chaney 1999). Although the mechanism responsible for increased GnT-V in cancer is not clear, this may be due in part to enhanced transcription of the GnT-V gene. For example, studies have revealed that the promotor region for the GnT-V gene contains a *src*-responsive element. In addition, this promoter contains binding sites for AP1 and Ets transcription factors (Pierce *et al*., 1997; Buckhaults *et al.,* 1997) whose activity is enhanced upon signaling of the Ras-Raf-MAPK pathway, which is upregulated in a number of cancers (Medema and Bos, 1993).

One approach to examining the contribution of GnT-V to metastasis is to examine the effect of reduced expression of β1-6 branched N-linked chains on the cell surface. Reduced expression of these glycans can be achieved by treating cells with swainsonine, a competitive inhibitor of Golgi α-mannosidase II which blocks the N-linked glycan synthesis pathway prior to initiation of β1-6 branching (Elbein, 1987). Swainsonine effectively prevents the formation of β1-6 branching, resulting in reduced binding of treated cells to endothelial cells and inhibition of tumor cell invasion *in vitro* (Cornil *et al*., 1990; Yagel *et al*., 1989). Treatment of cancer cells with swainsonine also reduced the metastatic potential of cells *in vivo* since the extent of both experimental and spontaneous metastases was reduced following injection of treated cells into mice (Humphries *et al.*, 1986). Other studies demonstrated that swainsonine-treated cells display increased production of TIMP-1 and decreased expression of MMP-2, both of which were hypothesized to reduce the invasive potential of treated cells (Korczak and Dennis, 1993; Seftor *et al*., 1991). These results suggested that β1-6 branched N-linked oligosaccharides may contribute to metastasis by mediating the expression of genes that contribute to enhanced metastatic potential. These effects could be mediated by altering receptor/ligand interactions on the cell surface, which would affect cellular signaling events and subsequent activation of transcription of a subset of genes.

An alternate approach to determine the contribution of GnT-V to metastasis involved examining the development of mammary tumors in GnT-V (a.k.a. Mgat5) knock-out mice. These studies revealed that Mgat5-/- mice developed tumors at a reduced rate and exhibited reduced incidence of lung metastases in the absence of GnT-V expression. After isolation of mammary tumor cells from Mgat5-/- mice, it was revealed that they exhibit reduced ability to develop focal adhesions, which may account for the reduced metastatic potential of these cells (Granovsky *et al*., 2000). Thus, the expression of β1-6 branched N-linked oligosaccharides appears to play a role in mediating receptor-ligand interactions, which in turn results in altered signaling pathways leading to reduced formation of focal adhesions and subsequent cell motility.

In light of these findings, it may be desirable to decrease the expression of β1-6 branched N-linked oligosaccharides on glycoconjugates produced by cancer cells. To

this end, patients suffering from cancer were treated with swainsonine in clinical trials. Treatment with swainsonine showed encouraging results, including low toxicity and tumor shrinkage in some patients (Goss *et al*., 1994; reviewed in Dennis *et al.,* 1999b).

2.3.2.3. Poly-N-acetyllactosamine

Poly-N-acetyllactosamine structures can be found on glycolipids as well as glycoproteins bearing both O-linked or N-linked oligosaccharides. Poly-N-acetyllactosamine structures are attached to core 2 or 4 structures of O-linked glycans or are attached preferentially to the β1-6 branch of N-linked oligosaccharides (Figure 2.10) (van den Eijnden *et al*., 1988; Ujita *et al.,* 2000). There are two types of poly-N-acetyllactosamine structures. Type I poly-N-acetyllactosamine chains consist of repeating units of Galβ1-3GlcNAc and are synthesized by β-1,3-N-acetylglucosaminyltransferases whereas type II chains consist of repeating units of Galβ1-4GlcNAc and are synthesized by β-1,4-N-acetylglucosaminyltransferases (reviewed in Zhou, 2003; Togayachi *et al.,* 2001). Recently, Ishida *et al*. (2005) identified a glycosyltransferase β−1,3-N-acetylglucosaminyltransferase-8, which catalyzes the biosynthesis of poly-N-acetyllactosamine structures on β1-6 branched N-linked oligosaccharides. Levels of this enzyme are elevated in human colorectal cancer tissue compared to normal colonic mucosa. After the elongation of glycan chains by poly-N-acetyllactosamine structures, further modifications are made at the terminal ends of these chains, as described below.

2.3.2.4 Alterations to terminal glycosylation

Terminal carbohydrate structures on oligosaccharides can serve as ligands for a number of lectins, which interact with specific carbohydrate structures and mediate many of the effects associated with glycosylation (reviewed in Gorelik *et al.,* 2001). These terminal structures are found on both glycosphingolipids as well as glycoproteins modified by either N-linked or O-linked glycans. The aberrant expression of terminal glycan structures is often associated with cancer progression and metastasis (Yogeeswaran and Salk, 1981).

2.3.2.4.1 Sialylation

Sialylation of N-linked, O-linked and glycosphingolipids results in the addition of negatively charged sialic acid residues by sialyltransferases. Elevated levels of sialyltransferases have been observed in a number of cancers (Yang *et al.,* 1994). The presence of sialic acid residues at the ends of carbohydrate branches correlates with enhanced malignancy in experimental metastasis models (Yogeeswaran and Salk, 1981). For example, elevated sialylation of glycosphingolipids has been observed in a number of colorectal carcinoma tumors (Kudo *et al*., 1998).

As previously mentioned, the metastatic potential of cells is associated with increased expression of β1-6 branched N-linked oligosaccharides which serve as scaffold for the addition of poly-N-acetyllactosamine repeats and sialic acid. Studies have shown that elevated levels of β1-6 branching resulted in the presence of more poly-N-acetyllactosamine chains and enhanced capping of termini with sialic acid residues. This in turn increased the metastatic potential of cancer cells (Dennis *et al*., 1987). In support of this, inhibition of β1-6 branching led to reduced levels of poly-N-acetyllactosamine and a reduction in the overall sialylation of cells, decreasing motility and invasiveness (Yoshimura *et al.*, 1995). The enzyme responsible for the addition of sialic acid residues to the terminal ends of β1-6 branched N-linked oligosaccharides is the α2-6 sialyltransferase ST6Gal-I. The elevated expression of ST6Gal-I and subsequent increase in sialylation has been observed in a number of human cancers and is associated with poor prognosis in patients suffering from colorectal or breast cancer (Recchi *et al*., 1998; Lise *et al*., 2000).

Elevated sialylation has also been observed at the ends of elongated chains such as poly-N-acetyllactosamine or at the end of short branches of O-linked glycans (reviewed in Brockhausen, 1999). For example, the elevated presence of shorter and more heavily sialylated O-glycans on the MUC1 mucin protein has been observed in breast cancer (Lloyd *et al.,* 1996). In addition, sialylation of the Tn O-linked antigen has also been identified as a tumor-associated carbohydrate structure as it was observed on a number of glycoproteins isolated from tumors, but not from normal mucosa (Thor *et al*., 1986). Furthermore, loss of sialylation following treatment of cells with sialidase

which removes terminal sialic acid residues, resulted in the generation of cells with decreased metastatic potential (Finne *et al.*, 1982).

2.3.2.4.2 Blood group antigens

Other examples of terminal glycosylation include the addition of blood group antigens which consist of histo-blood group ABH antigens and tissue-specific Lewis antigens (Figure 2.12). These carbohydrate antigens are aberrantly expressed by cancer cells and result in altered cellular function, usually through interaction with endogenous lectins (reviewed in Gorelik *et al.,* 2001).

Lewis antigens are carbohydrate structures which are added to the termini of glycan chains of both glycoproteins and glycolipids (Figure 2.10). Lewis antigens are added to carbohydrate structures containing elongated poly-N-acetyllactosamine repeats. Lewis ^{x} (Le^{x}), Lewis ^{y} (Le^{y}) and sialyl-Lewis ^{x}(sLe^{x}) are added to poly-N-acetyllactosamine type II chains whereas Lewis ^{a}(L^{a}) and sialyl-Lewis ^{a} (sLe^{a}) are found terminating type I poly-N-acetyllactosamine chains (reviewed in Gorelik *et al.*, 2001). The enhanced expression of Lewis blood group antigens has been observed in a number of human carcinomas and often correlated with advanced malignancy. For example, there is a positive correlation between expression of sLe^{x} and liver metastasis and poor prognosis in colorectal cancer patients (Nakamori *et al.*, 1993). As previously mentioned, enhanced sialylation of cell surface carbohydrates has been implicated in the establishment of metastatic disease. Not surprisingly, elevated expression of sLe^{x} and sLe^{a} carbohydrate antigens on both N-linked and O-linked glycans have been implicated in enhanced metastatic potential of cancer cells (Shimodaira *et al.,* 1997).

Histo-blood group ABH antigens are expressed on the surface of red blood cells and dictate blood-type (Figure 2.10). During oncogenic transformation, tumor cells express altered histo-blood group antigens at the ends of poly-N-acetyllactosamine chains affecting metastatic potential (Hakomori, 1999). For example, loss of A and B antigen expression has been observed in a number of malignancies and is associated with reduced survival in these patients (Mandel *et al.*, 1992). Loss of these carbohydrate antigens is hypothesized to result in enhanced binding of growth factors to cell surface receptors resulting in elevated cell proliferation and motility. Altered expression of

histo-blood group H antigen has also been observed in cancer. In fact, colorectal carcinoma tumors have been observed to express elevated levels of histo-blood group H antigen (Yang *et al.,* 1994). Cancer cells expressing elevated levels of histo-blood group H carbohydrate antigen are more aggressive and tumorigenic than those expressing reduced levels (Goupille *et al*., 1997).

2.3.2.5 Lectins

The presence of carbohydrates on glycoproteins or glycolipids not only directly affects the function of the macromolecule they are attached to (Opdenaker *et al*., 1993; Chammas *et al*., 1991; Kornfeld, 1980), but they also have the ability to bind lectins. Lectins are proteins that bind to specific carbohydrate structures and may mediate the effects that are associated with the presence of carbohydrates. Initially lectins were isolated from a number of different plant species, however, a number of mammalian lectins have been identified in recent years. Some examples of endogenous human lectins include C-type lectins and S-type lectins.

C-type lectins are calcium-dependent cell surface lectins such as selectins. Selectins are normally involved in tissue injury or inflammation, but have been implicated in the metastatic spread of cancer cells (Fukada, 1996). Examples of selectins include L-selectin which is expressed on the surface of leukocytes, E-selectin which is expressed mainly on endothelial cells and P-selectin which is expressed on endothelial cells as well as platelets (Fukada, 1996).

Carbohydrate ligands for selectins consist of terminal sialylated Lewis antigens such as sLe^x and sLe^a (Fukada *et al.,* 1999). The ability of cancer cells to establish metastasis at a secondary organ requires that the tumor cells be able to adhere to the vascular endothelium at this secondary sight. This adhesion is mediated by the interaction between selectins such as E-selectin or P-selectin expressed by the vascular endothelium and sialylated Lewis antigens expressed on the tumor cell surface (Biacone *et al.,* 1996). In order to enhance the adherence of tumor cells to the vasculature at the secondary site, tumor cells stimulate the release of cytokines from endothelial cells resulting in elevated expression of E-selectin by these endothelial cells (Khatib *et al.,* 1999). In support of the role of sialylated Lewis antigens in the establishment of distant

metastasis, studies have shown that blocking the synthesis of sLe^x and sLe^a by over expressing a fucosyltransferase that competes with Lewis antigen synthesis prevented liver colonization by colorectal carcinomas (Weston *et al.,* 1999). It was suggested that a decrease in metastatic potential of the cancer cells resulted from decreased adhesiveness of cancer cells expressing reduced sialylated Lewis antigens and E-selectin (reviewed in Brockhausen, 1999).

In addition to playing a role in mediating adhesion to the vascular endothelium, selectin-ligand interactions have also been implicated in maintaining homotypic interactions between tumor cells, a necessary step for tumor cell embolization and survival in the vasculature. The expression of both selectins and their sialylated Lewis antigen ligands have been found on the cell surface of cancer cells, suggesting that this dual expression may be involved in the clustering of cells (Hittelet *et al.*, 2003a). In support of this hypothesis, some tumor cells express P-selectin, which is normally present on the surface of activated platelets or endothelial cells. The presence of P-selectin on the tumor cell surface may also enhance embolization by mediating interactions between platelets and tumor cells (Stone and Wagner, 1993).

In addition to selectins, other types of endogenous lectins exist and play important roles in a number of cellular functions. For example, galectins are S-type lectins that have been implicated in cell adhesion, apoptosis, cell growth, inflammation, embryogenesis, immune function and tumor metastasis formation (reviewed in Gorelik, 2001). Fourteen mammalian galectins have been identified to date, all of which bind to type I or type II N-acetyllactosamine structures (reviewed in Hughes, 2001). Binding of galectins to their carbohydrate ligands is mediated by a carbohydrate recognition domain (CRD). Most galectins have only one CRD, however galectin-4, -6, -8, -9 and -12 have two such domains (reviewed in Dumic *et al.*, 2006). Galectins can function as monomers, dimers or oligomers and they have been detected in the nucleus, cytoplasm as well as extracellularly. Extension of glycan chains with poly-N-acetyllactosamine and capping with terminal carbohydrate structures may alter the binding affinity of galectin family members. For example, galectin-3 has a high affinity for the human blood group A epitope Galα1,3[Fuc1,2]Galβ1,4GlcNAc however galectin-1 does not (Sparrow *et al.*, 1987).

Galectins-1 and -3 have been the subject of numerous studies as both have been implicated in a number of cancers (reviewed in van den Brûle *et al*., 2004). Galectin-1 is a 14 kDa homodimer that binds to carbohydrate structures on glycoproteins including fibronectin, laminin and lysosome-associated membrane proteins (LAMPs) (reviewed in Gorelik, 2001). Expression of galectin-1 has been associated with the induction of apoptosis (Perillo *et al.,* 1996). Studies have revealed elevated levels of galectin-1 in a number of cancers compared to normal tissues (reviewed in van den Brûle *et al*., 2004). In addition, elevated levels of cytoplasmic galectin-1 were associated with increasing dysplasia and malignancy in human colorectal carcinoma specimens (Hittelet *et al.,* 2003b).

Galectin-3 is a 31 kDa β-galactoside binding protein composed of three domains including an amino terminal domain, a repetitive collagen-like domain and a carboxy-terminal domain which contains the carbohydrate recognition domain (CRD) (Barondes *et al.,* 1994). Galectin-3 can function as a monomer, however self-association mediated by the amino terminal domains of monomers results in oligomerization and enhances the binding affinity to ligands (Barboni *et al*., 2000). Galectin-3 has been identified in the nucleus, cytoplasm as well as extracellularly. Galectin-3 binds to carbohydrate structures on glycoproteins such as laminin, Mac-2 binding protein/tumor-associated antigen 90K (TAA90K), integrins, CEA and mucin and has been identified as a substrate for MMP-2 and MMP-9 (Sasaki *et al.*, 1998; Ohannesian *et al*., 1995; Ochieng *et al*., 1994). Cleavage of galectin-3 by MMPs results in the generation of cleavage products that can bind more tightly to glycan ligands (Ochieng *et al*., 1998).

Galectin-3 is ubiquitously expressed by epithelial cells and immune cells, including surface expression on activated macrophages (reviewed in Dumic *et al.,* 2006). Elevated expression of galectin-3 has been associated with tumor progression in a number of cancers. For example, elevated levels of galectin-3 in colorectal cancer have correlated with Duke's staging and poor survival (Lee *et al.*, 1991). Furthermore, studies have shown that transfection of non-tumorigenic breast carcinoma cell lines with the cDNA encoding galectin-3 results in enhanced tumorigenicity following injection into nude mice (Nangia-Makker *et al.,* 1995).

Unlike other galectins which have been shown to induce apoptosis, galectin-3 actually exhibits an anti-apoptotic effect (Akahani *et al.,* 1997). Studies have revealed that the anti-apoptotic effect of galectin-3 is mediated by a NWGR motif. This motif has also been identified in the protein Bcl-2 and is required for mediation of its anti-apoptotic effects (Akahani *et al*., 1997). Thus, galectin-3 may contribute to tumor progression by inhibiting apoptosis of tumor cells. The anti-apoptotic function of galectin-3 is regulated by its phosphorylation state. Galectin-3 present in the cytoplasm becomes phosphorylated by casein kinase I resulting in the translocation of galectin-3 into the nucleus where it elicits its anti-apoptotic effects and protects cells from death induced by chemotherapeutic agents (Takenada *et al.,* 2004). The anti-apoptotic effect of galectin-3 is believed to be mediated through its interaction with the mitrochondrial membrane, thereby preventing the release of cytochrome *c* and subsequent activation of the caspase cascade (Yu *et al.,* 2002). Thus, galectin-3 may contribute to tumorigenesis by inhibiting apoptosis.

Enhanced expression of galectin-3 by cancer cells may result in enhanced tumorigenicity by stimulating cellular proliferation. Shimura *et al*. (2004) have identified β-catenin as a ligand for galectin-3. Upon translocation to the nucleus, β-catenin and galectin-3 co-localize and activate the transcription factor TCF-4 resulting in transcriptional activation of genes that enhance cellular proliferation such as cyclin D1, cyclin A, cyclin E, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. Galectin-3 may also stimulate cellular proliferation by binding to the activated *ras* oncogene product resulting in the activation of the Ras-MAP kinase pathway (Shalom-Feuerstein *et al.,* 2005).

Galectin-3 may also enhance tumorigenesis by stimulating angiogenesis. Galectin-3 was able to stimulate *in vitro* capillary tube formation as well as the chemotaxis of human umbilical vascular endothelial cells (HUVEC). In addition, galectin-3 was able to initiate *in vivo* neovascularization. These effects were shown to be inhibitable by lactose, which competes with ligands for galectin-3 binding, implicating galectin-3 binding to glycan structures in these processes (Nangia-Makker *et al.,* 2000).

In addition to enhancing tumorigenesis, galectin-3 has also been implicated in mediating cell adhesion as well as cell-cell interactions. For example, breast cancer cell

lines that have been transfected with a cDNA encoding galectin-3 exhibited enhanced adhesion to ECM proteins including laminin and collagen IV (Warfield *et al.,* 1997). In addition, these cells exhibited enhanced invasion through a reconstituted basement membrane known as Matrigel in *in vitro* invasion assays and was associated with an increased expression of α6β1 integrin (Warfield *et al.,* 1997). Furthermore, increased expression of galectin-3 in poorly metastatic cell lines resulted in enhanced metastatic potential (Raz *et al.,* 1990).

Since aggregation of cells is an important requirement for embolization and survival in the vasculature, leading to increased incidence of metastases (Liotta *et al.,* 1976), it was hypothesized that galectin-3 may enhance the spread of cancer cells by establishing cell-cell contacts. The addition of asialofetuin, a glycoprotein bearing poly-N-acetyllactosamine structures, resulted in galectin-3-mediated aggregation of cells (Inohara and Raz, 1995). Similarly, addition of Mac-2-binding protein/TAA90K, which is a major ligand for galectin-3, resulted in the homotypic aggregation of melanoma cells, suggesting that Mac-2 binding protein/TAA90K and galectin-3 interact on the cell surface to promote the formation of homotypic interactions (Inohara *et al.,* 1996). The cellular aggregates formed in both of these studies were inhibited by lactose, indicating that galectin-3 binding to glycan structures on these glycoproteins was responsible for the mediation of aggregate formation. It is through the formation of homotypic interactions that galectin-3 is hypothesized to enhance emboli formation and prevent anoikis resulting in enhanced metastatic potential (Kim *et al.,* 1999).

Galectin-3 may also contribute to metastasis by enhancing cell motility through its interaction with glycans expressed on integrin receptors. Galectin-3 interacts with glycoconjugates bearing poly-N-acetyllactosamine repeats that are added preferentially to β1-6 branched N-linked oligosaccharides generated by GnT-V (a.k.a. Mgat5). For example, recent research has examined the effect of Mgat5 activity on the formation of focal adhesions required for cell motility. Mammary epithelial tumor cells isolated from Mgat5-/- mice which lack β1-6 branched oligosaccharides exhibited impaired fibronectin fibrillogenesis as well as fibronectin-dependent cell spreading. These results were also observed in mammary tumor cells from Mgat5+/+ mice that were treated with either swainsonine or competitive inhibitors of galectin-3 binding. Further

analysis revealed that galectin-3 interacts with Mgat5 modified N-glycans expressed on α5β1 integrin receptors involved in fibronectin binding. Binding of galectin-3 initiated integrin activation resulting in clustering of cell surface integrins as well as receptor tyrosine kinases and subsequent translocation to fibrillar adhesions. In addition, activation of cell surface receptors initiated signaling events which led to the activation of focal adhesion kinase (FAK), phosphatidylinositol 3 kinase (PI3K) and increased turnover of F-actin. Thus, binding of galectin-3 to Mgat5-modified N-glycans on α5β1 integrin resulted in fibronectin polymerization and enhanced tumor cell mobility through stimulation of focal adhesion remodeling (Lagana *et al*., 2006). Another study revealed that galectin-3 also interacts with Mgat5 modified N-glycans on cell surface cytokine receptors including epidermal growth factor and transforming growth factor β receptors, delaying their removal by endocytosis (Partridge *et al*., 2004).

2.4 GLYCOPROTEINS ASSOCIATED WITH CANCER

The identification of novel targets to aid in the detection, prevention and treatment of cancer is a challenging task. Since altered glycosylation of lipids and proteins has been implicated in tumor progression and metastasis, identification of glycoconjugates modified by cancer-associated glycan chains may uncover novel cancer markers and provide new approaches for cancer management.

2.4.1 The role of glycoproteins modified by β1-6 branched N-linked oligosaccharides

Cancer cells expressing elevated levels of β1-6 branched N-linked oligosaccharides have enhanced metastatic potential (Dennis *et al*., 1987; Fernandes *et al.,* 1991). The identification of glycoproteins that are modified with these cancer-associated carbohydrates may provide insight about their contribution to increased invasion and metastasis. In addition, the identification of proteins modified by β1-6 branched oligosaccharides may uncover tumor markers that will aid in the diagnosis and/or treatment of cancer. For example, carcinoembryonic antigen (CEA) has been identified as a major carrier of β1-6 branched N-linked oligosaccharides (Chandrasekaran *et al.,* 1983 Wojciechowicz et al., 2000). Serum levels of CEA are

used to monitor prognosis of patients with colon, breast or lung cancer after surgery. Elevated serum levels of CEA are a factor for poor prognosis and cancer recurrence (Goldenberg *et al.,* 1981). CEA is expressed in adenocarcinomas of the digestive tract and fetal gastrointestinal tissue where it mediates homotypic cell adhesion (Benchimol *et al.,* 1989). Overexpression of CEA has been observed in a number of cancers including colorectal cancer where it is implicated in the establishment of hepatic metastases. Specifically, tumor cells expressing CEA on their surface bind to receptors on hepatic cells, stimulating the release of cytokines that increase ICAM-1 expression on vascular endothelium, which enhances the adhesion of cancer cells (Wagner *et al.,* 1992). The presence of oncodevelopmental carbohydrates such as β1-6 branching on CEA produced by cancer cells may result in enhanced cell-cell mediated adhesion compared to CEA produced by normal cells (Charbonneau and Stanners, 1999).

Other examples of glycoproteins bearing cancer-associated carbohydrates are the lysosomal membrane associated proteins -1 and -2 (Lamp-1 and Lamp-2), which are normally localized in the membrane of lysosomes. However, they have also been observed on the cell surface of cancer cells (Hughes and August 1981; Mane *et al*., 1989). Both Lamp-1 and Lamp-2 are heavily glycosylated and have been identified as carriers of β1-6 branched N-linked oligosaccharides (Laferté and Dennis, 1989; Ochwat *et al*., 2004). The presence of sialic acid and poly-N-acetyllactosamine on β1-6 branches of N-linked oligosaccharides resulted in decreased binding of Lamp-1 to ECM proteins and increased motility and invasiveness (Laferté and Dennis, 1988). The overexpression of Lamp-1 by tumor cells has been associated with enhanced tumor invasion in pancreatic carcinomas (Künzli *et al.,* 2001). In addition, elevated expression of Lamp-1 and Lamp-2 by colorectal cancer cell lines was associated with enhanced metastatic potential of these cells (Saitoh *et al*., 1992).

Integrins are another example of glycoproteins that are carriers for β1-6 branched N-linked oligosaccharides. Integrins are heterodimeric cell surface receptors that are composed of both α and β subunits (reviewed in Hood and Cheresh, 2002). These receptors bind to extracellular ligands and mediate cell-cell as well as cell-ECM interactions. The increased expression of β1-6 branched N-linked oligosaccharides on integrins has been observed to result in altered cell-cell and cell-ECM interactions

resulting in cells with enhanced motility and metastatic potential (reviewed in Gu and Taniguchi, 2004) . Integrin subunits that have been identified as targets of GnT-V and carriers of β1-6 branched N-linked glycans include α2, α3, α5 and β1 integrin subunits (Prokopishyn *et al.,*1999; Ochwat *et al.,* 2004). For example, elevated expression of β1-6 branched N-linked oligosaccharides on β1 integrin subunits resulted in the inhibition of α5β1-mediated spreading of cells on fibronectin, resulting in enhanced migration and invasion. It was observed that enhanced motility was the result of decreased clustering of α5β1 integrins on the cell surface as a result of elevated levels of β1-6 branching (Zheng *et al*., 1994).

2.4.1.1 Tumor-associated antigen 90K

In an effort to identify glycoproteins that contribute to colon cancer progression, Laferté and Loh (1992) identified the tumor-associated antigen 90K (TAA90K; a.k.a Mac-2-binding protein; galectin-3-binding protein) as a major carrier of β1-6 branched N-linked oligosaccharides. In addition, Ochwat *et al.,* (2004) identified TAA90K as a carrier of β1-6 branching in melanoma cells. These findings were recently confirmed by Kim *et al.* (2006) who identified TAA90K as a substrate for N-acetylglucosaminyltransferase V activity, the enzyme responsible for catalyzing β1-6 branching.

In an attempt to examine the regulation of TAA90K expression, the promoter for the TAA90K gene has been analyzed (Brakebusch *et al*., 1997, 1999). The gene for TAA90K was found to be 8.8-kilobase pairs consisting of 6 exons and was found to be localized on chromosome 11, region E (Brakebusch *et al*. 1997). Analysis of the gene for TAA90K revealed a TATA-less promoter with several unique features. For example, the promoter was found to be neither GC-rich nor was it dependent on SP1 sites. Further study revealed the presence of one major transcriptional start site as well as a minimal promoter, which contains an interferon responsive element (IRF-E) (Brakebusch *et al.,* 1999). In support of this, TAA90K expression was found to be induced by interferons (IFNs) –α and –γ (Marth *et al*., 1994; Brackebusch *et al.,* 1997).

Following the isolation and analysis of the cDNA for TAA90K, it was revealed that the open reading frame encodes a 585 amino acid protein (Koths *et al*., 1993;

Ullrich *et al.,* 1994). Based on the amino acid sequence of TAA90K, four putative domains were predicted (Müller *et al*., 1999). Figure 2.15 is a schematic diagram of the putative TAA90K domains including potential N-linked oligosaccharide sites and protease cleavage sites. The N-terminal domain 1 begins after the signal sequence (amino acids 19-133) and is homologous to the scavenger receptor cysteine-rich domain (SRCR) found in members of the macrophage scavenger receptor superfamily (Pearson *et al.,* 1996). Domain 2 spans amino acids 127-250 and corresponds to a putative BTB/POZ domain (*B*road complex, *t*ramtrack, *b*ric-a-brac/*po*xvirus and *z*inc finger) found in developmentally regulated zinc finger proteins and the kelch family of actin-associated proteins thought to mediate protein-protein interactions (Carim-Todd *et al*., 2001). Domain 3 consists of amino acids 251-408 which has homology with an IVR domain (intervening sequence between BTB and kelch) (Robinson *et al*., 1997). The remaining amino acid sequence consists of a 40 amino acid link region followed by domain 4 (amino acids 451-585) whose sequence is consistent with a globular fold. It has been shown that domains 2, 3 and 4 are important in forming the oligomeric complex and mediating adhesion to extracellular matrix proteins (Müller *et al*., 1998; Hellstern *et al*., 2002).

Although the amino acid sequence of TAA90K predicts a polypeptide size of approximately 60 kDa (Koths *et al*., 1993; Laferté *et al.,* 2000), TAA90K is synthesized initially as a 74 kDa glycoprotein modified with high-mannose type oligosaccharides (Laferté and Loh, 1992; Koths *et al*., 1993). Further processing of the glycans in the Golgi results in the generation of a 90-100 kDa glycoprotein modified with complex-type N-linked oligosaccharides which is secreted extracellularly and forms large homopolymers that assemble into ring-like structures (Sasaki *et al.,* 1998). Although TAA90K was found to be heavily modified by oligosaccharide structures, no O-linked oligosaccharides were detected (Laferté and Loh, 1992; Laferté *et al.,* 2000; Hellstern *et al.,* 2002).

Studies have identified cyclophilin-C associated protein (CyCAP) as the murine orthologue of TAA90K. CyCAP is a 77 kDa protein that shares 69% protein sequence identity with TAA90K (Figure 2.16) (Trahey and Weissman, 1999). It binds cyclophilin C, a peptidylprolyl cis-trans isomerase that is involved in mediating protein folding

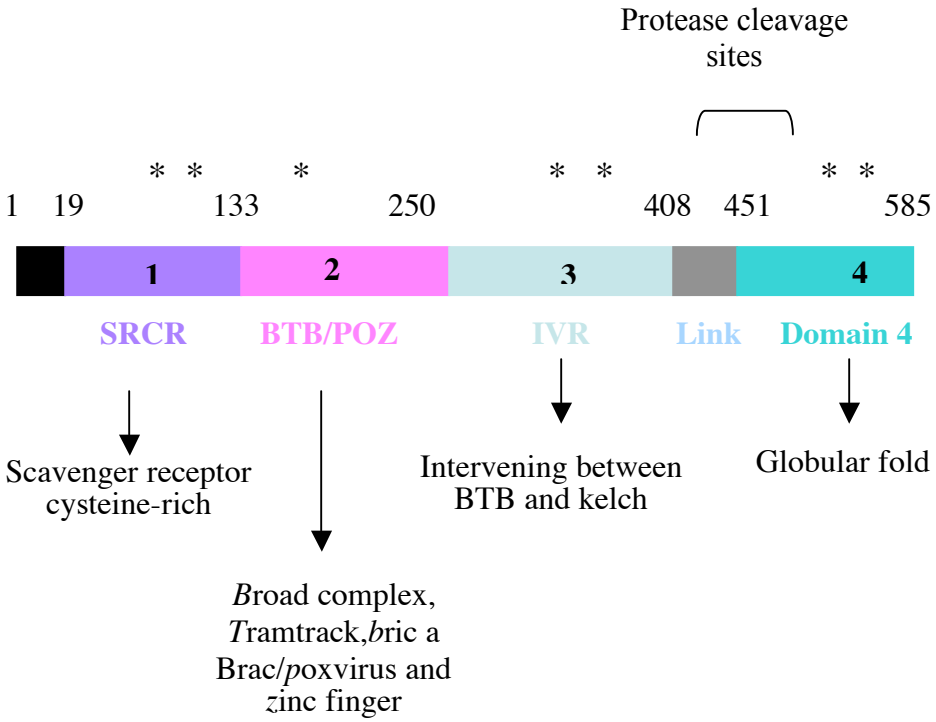


Figure 2.15: Schematic of the putative TAA90K domains and potential asparagine-linked glycosylation sites. The signal sequence is indicated in black. Putative domains are represented by boxes and the amino acid residues involved at the boundaries of the domains are indicated. Potential asparagine-linked glycosylation sites are indicated by asterisks (*). Protease cleavage sites for trypsin and plasmin are indicated.

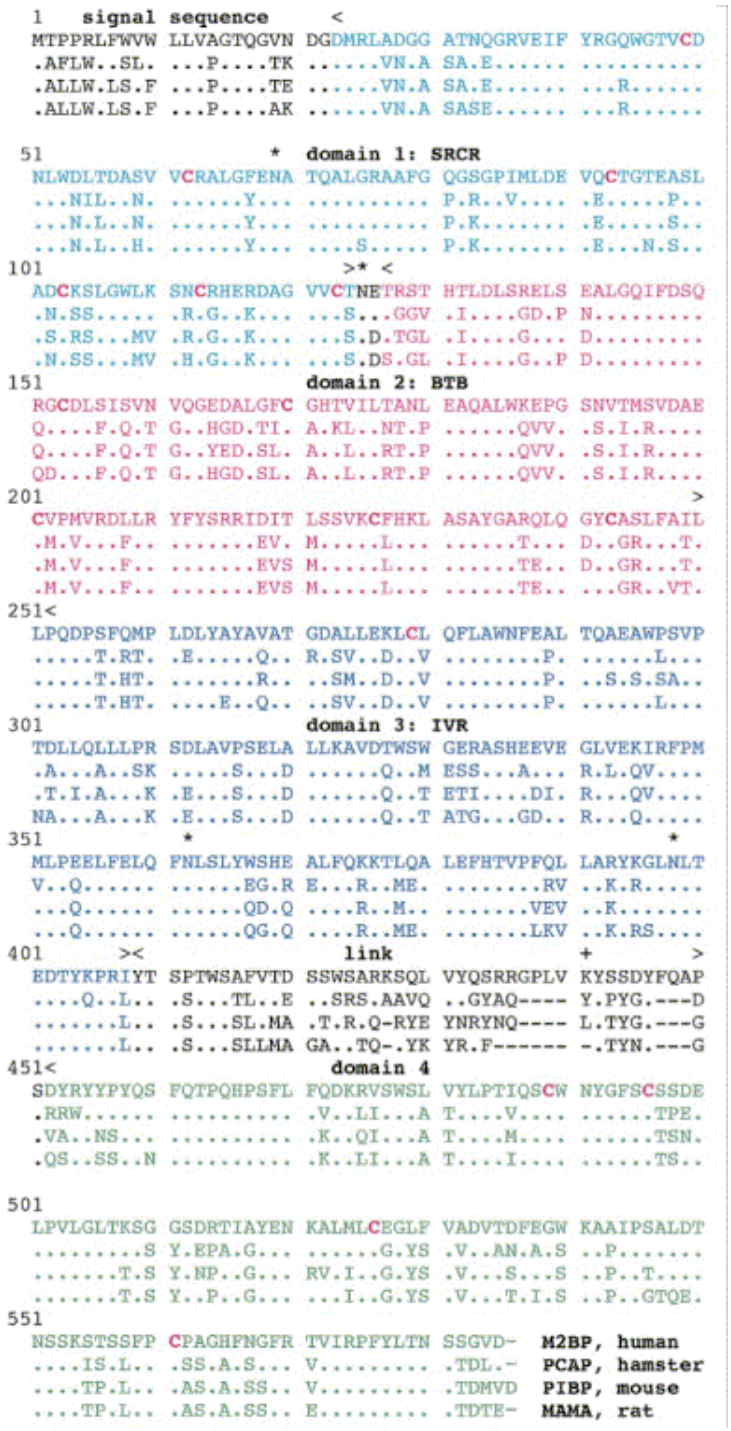


Figure 2.16: Alignment of amino acid sequences for human TAA90K/M2BP, Hamster PCAP, mouse CyCAP/PIBP and rat MAMA protein. The signal sequence and domains 1-4 are indicated. Putative glycosylation sites are marked with astericks (*) and plasmin cleavage site in TAA90K with a +. From Müller *et al.* (1999).

(Friedman *et al*., 1993). Both TAA90K and CyCAP share an amino-terminal signal sequence followed by a scavenger receptor cysteine-rich domain as well as several putative asparagine-linked glycosylation sites (Figure 2.15). Despite the sequence similarities between CyCAP and TAA90K, these two proteins exhibit differences in ligand binding. CyCAP binds to human and mouse cyclophilin C, however, it cannot bind to mouse or human Mac-2. On the other hand, TAA90K is unable to bind to either mouse or human cyclophilin C (Jalkanen *et al*., 2001). These biological differences may result from conformational differences between individual or multiple domains of these two proteins.

2.4.1.1.1 Expression of TAA90K in normal and cancerous tissues

TAA90K is a secreted glycoprotein that is present in normal human fluids including plasma, breast milk, saliva, tears and urine, a wide range of epithelial tissues (Iacobelli *et al*., 1986; Linsley *et al*., 1986; Koths *et al.,* 1993; Jallal *et al.,* 1995), but elevated in the serum of patients suffering from cancer (Linsley *et al*., 1986; Natoli *et al.,* 1993; Ullrich *et al*., 1994; Jallal *et al.,* 1995; Iacobelli *et al.,* 1986, 1993, 1994) and viral infections including acquired immunodeficiency virus and hepatitis C (Natoli *et al*., 1993; Artini *et al*., 1996).

Analysis of TAA90K expression by Northern blotting and immunohistochemistry has revealed elevated levels of TAA90K in pancreatic, breast lung, and gastric cancers (Fusco *et al.,* 1998; Künzli *et al.,* 2002; Marchetti *et al*., 2002; Ozaki *et al*., 2002; Park *et al*., 2006). In the cases of non-small cell lung cancer and malignant mesothelioma, TAA90K expression correlated with metastasis and predicted survival (Marchetti *et al*., 2002) or disease manifestation (Singhal *et al*., 2003), respectively. In breast cancer patients, serum levels $\geq$11μg/mL TAA90K correlated with poor prognosis (Iacobelli *et al*., 1994). However in this case, TAA90K was expressed by the tumor as well as by peripheral blood mononuclear cells, indicating that TAA90K may play a role in host anti-tumor immune responses (Fusco *et al*., 1998). TAA90K levels were also elevated in patients suffering from biliary tract carcinoma and when analyzed in combination with CA19-9, the current marker for this type of cancer, the diagnostic significance was improved compared to the use of either marker individually

(Koopman *et al*., 2004). Elevated levels of TAA90K may also have diagnostic significance for patients suffering from nasopharyngeal carcinoma (Wu *et al*., 2005).

2.4.1.1.2 Interaction with extracellular proteins

Although the function of TAA90K is not known, there is evidence to suggest that it may be involved in the modulation of cell adhesion. Sasaki *et al*. (1998) have shown that TAA90K is deposited in the extracellular space and is able to bind to extracellular matrix proteins including laminin-1, collagens and fibronectin as well as mediate cell adhesion in a β1-integrin-dependent manner. These data suggested that TAA90K may act as a cross-linking bridge between tumor cells and the extracellular matrix, thereby modulating tumor cell adhesion.

In addition to being modified by β1-6 linked oligosaccharides, TAA90K is also modified with poly-N-acetyllactosamine structures (repeating Galβ1-4GlcNAc units) (Laferté and Loh, 1992). These oligosaccharides are potential binding sites for galectin-3, a mammalian lectin and dominant ligand for TAA90K (Hughes, 2001). Binding of TAA90K to cell surface-associated galectin-3 via its domains 3 and 4 has been shown to cause tumor cell aggregation (Inohara *et al*., 1996). TAA90K is also a ligand for galectin-1 and is able to stimulate galectin-1 induced cellular aggregation (Tinari *et al.,* 2001). These results suggested that extracellular co-localization of TAA90K with galectin-3 and possibly galectin-1 may contribute to metastasis by enhancing tumor emboli formation.

TAA90K may also contribute to tumor progression by modulating the expression or function of MMPs, proteolytic enzymes shown to play a key role in tumor invasion and metastasis (reviewed in Nagase *et al*., 2006). Recent studies have shown that in addition to being a potential tumor marker for prostate cancer, TAA90K induced the expression of promatrilysin (proMMP-7) when it was added to the culture medium of the prostate cancer cell line LNCaP (Bair *et al.,* 2006). Other studies revealed that proMMP-7 expression in the LNCaP cell line is mediated by IL-1β induced IL-6 expression and signaling through STAT-3 (Maliner-Stratton *et al.,* 2001). Since proMMP-7 expression was blocked by both anti-TAA90K and anti-IL-6 antibodies

(Bair *et al*., 2006), this suggested that TAA90K may enhance the expression of proMMP-7 through the induction of IL-6 (Ullrich *et al*., 1994).

In addition to an effect on MMP expression, TAA90K may also have an effect on ADAMTS (a disintigrin and metalloproteinase with thrombospondin motifs), another family of metalloproteinases that has been shown to be involved in the restructuring of the ECM (Sandy *et al*., 2001). Recent studies employing proteomic screening to elucidate potential targets for ADAMTS1 activity have identified TAA90K as a potential substrate for this enzyme. In support of this hypothesis, overexpression of ADAMTS1 in human embryonic kidney 293T cells was associated with reduced levels of TAA90K in the medium (Canals *et al*., 2006). The putative cleavage of TAA90K by ADAMTS1 and perhaps other metalloproteinases may alter the function of TAA90K, possibly through changes in three-dimensional structure. In this regard, cleavage of TAA90K in the linker region by trypsin and plasmin has been observed (Hellstern *et al*., 2002; Sasaki *et al.,* 1998).

2.4.1.1.3 Immunomodulation

In addition to binding to a number of extracellular proteins and mediating cellular adhesion, TAA90K has also been implicated in modulating immune responses. For example, elevated TAA90K levels in the serum of breast cancer patients were found to be the result of increased production of TAA90K by peripheral blood mononuclear cells (PBMC), suggesting that TAA90K may modulate immune responses (Fusco *et al*., 1998). Furthermore, the addition of TAA90K to PBMC led to the activation of T-cells, natural killer (NK) and lymphokine-activated killer (LAK) cells by stimulating PBMC to release cytokines including IL-1, IL-2 and IL-6 (Ullrich *et al*., 1994; Powell *et al*., 1995). TAA90K was also found to contain a SRCR domain which is found in members of the macrophage scavenger receptor superfamily previously implicated in development and regulation of the immune system, including complement factor I, CD5 and CD6 (Goldberger *et al.,* 1987; Kodama *et al*., 1990; Aruffo *et al.,* 1991). In addition, Jallal *et al.* (1995) revealed that the subcutaneous injection of TAA90K-expressing tumors in nude mice resulted in elevated VCAM-1 and ICAM-1 expression on endothelial cells of blood vessels resulting in enhanced infiltration of leukocytes.

These results confirm previous studies by Powell *et al.,* (1995) demonstrating that addition of purified TAA90K to a human monocytic cell line resulted in the upregulation of ICAM-1 expression.

In other studies, Trahey and Weissman (1999) demonstrated that genetic knock-out mice deficient in CyCAP expression were found to be more sensitive to the effects of bacterial endotoxin than wildtype mice, suggesting that CyCAP and possibly TAA90K are involved in the regulation of endotoxin and pro-inflammatory responses *in vivo*. In support of this, TAA90K was able to bind to the cell surface protein CD14 when its ligand bacterial lipopolysaccharide (LPS, endotoxin) was bound (Yu and Wright, 1995). Thus, TAA90K may play a role in modulating the pro-inflammatory response which is characterized by elevated secretion of cytokines including tumor necrosis factor-α (TNF-α), Il-1β, IL-6 and IL-8 and has been implicated in the development of some cancers, including stomach and colon (Coussens and Werb, 2002).

Recent studies have revealed that the expression of CyCAP is elevated in keratinocytes as well as infiltrating immune cells during the process of wound repair in mice, suggesting a role in inflammation and subsequent wound healing. In support of this is the finding that wound healing is impaired in CyCAP null mice (Kong *et al.,* 2007). Matrix metalloproteinases including MMP-13 are involved in remodeling of the ECM during processes such as wound repair and have also been implicated in the progression of cancer and other diseases (Moore *et al*., 2000). Previous work revealed that the expression of MMP-13 can be upregulated by the binding of fibronectin fragments to cell surface receptors (Stanton *et al*., 2002). Recently, it was revealed that fibroblasts from wildtype mice express elevated levels of MMP-13 following treatment with fibronectin fragments, whereas CyCAP null mice do not (Kong *et al*., 2004). These results would suggest that CyCAP is involved in mediating fibronectin fragment-induced expression of MMP-13, possibly through interaction with fibronectin or integrin receptors.

In summary, TAA90K was identified as a major carrier of cancer-associated carbohydrates and appears to be a marker for a number of types of cancer. Although the function of TAA90K is not well understood, studies have revealed that it is a multi-

adhesive protein with the potential to interact with ECM proteins, galectins and MMPs suggesting a role in modulating tumor cell adhesion to the ECM and galectins. In addition, TAA90K may also function in immunomodulation by stimulating the release of cytokines and activating accessory cells of the immune system.

3.0 RATIONALE AND OBJECTIVES

The elevated expression of β1-6 branched N-linked oligosaccharides by cancer cells has correlated with enhanced metastatic potential in both breast and colon cancer. Thus, the identification of glycoproteins carrying these cancer-associated glycans may contribute to our understanding of the role of these glycans in cancer progression. Previous work in our laboratory identified TAA90K, secreted by colon cancer cell lines, as a carrier of these cancer-associated carbohydrates. Elevated expression of TAA90K has been shown to correlate with cancer progression and poor prognosis in a number of cancers. Although elevated levels of TAA90K have been observed in a number of cancers and viral infections, its role has yet to be elucidated. Thus, this thesis examines the expression of TAA90K in human colon cancer and attempts to clarify its role in cancer progression.

The specific objectives of these studies are as follows:

(1) To examine the expression of TAA90K in human colon tumors and normal colonic mucosa
(2) To examine the expression of TAA90K in human colon cancer cell lines
(3) To purify TAA90K from human colon cancer cell lines for use in functional studies
(4) To examine the interaction between TAA90K and ECM proteins, galectins and MMPs
(5) To examine the effect of TAA90K on MMP expression by colon cancer cells
(6) To examine the effect of TAA90K cleavage on its function, including binding to ECM proteins and galectins
(7) To examine the role of TAA90K tumor cell adhesion, invasion and migration

4.0 MATERIALS AND METHODS

4.1 Cell lines

The human colon carcinoma cell lines HT-29 (HTB 38), SW403 (CCL 230) SW620 (CCL 227), LoVo (CCL 229), LS123 (CCL 255), Caco-2 (HTB 37), the normal rat fibroblast cell line Rat2 (CRL 1764) and the normal rat small intestinal epithelial cell line IEC-6 (CRL 1592) as well as COS-1 (CRL 1650) and the African green monkey cell line CV-1 (CCL-70) were obtained from the American Type Culture Collection (A.T.C.C., Rockville, MD). The human colon carcinoma cell line KM20C and the human breast epithelial cell line HBL-100 (HTB 124, ATCC) were obtained from Drs. I. J. Fidler (MD Anderson Cancer Centre, Houston, TX) and S. Carlsen (Saskatoon Cancer Research Unit, Saskatoon, SK), respectively. Cells were cultured in complete medium consisting of Dulbecco's minimum essential medium (DMEM, high glucose) supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids and 2% MEM vitamin solution (Invitrogen, Burlington, ON). In routine testing, all of the cell lines used were found to be free of mycoplasma contamination. For purification of TAA90K from HT-29-conditioned medium, cells were cultured in complete F12/DMEM serum-free medium (Invitrogen, Burlington, ON) supplemented with 2 μg/mL insulin, 20 ng/mL epidermal growth factor, 13 ng/mL triiodothyronine and 2 μg/mL transferrin (Sigma, Oakville, ON).

4.2 Construction and expression of His-TAA90K

The entire open reading frame (ORF) of TAA90K was inserted in-frame behind the poly-histidine tag into the expression vector pRsetB (Invitrogen, Burlington, ON) for expression of His-TAA90K as described (Laferté *et al*., 2000). Briefly, the entire open reading frame for TAA90K was reconstructed by inserting the 1.174 kb *Kpn*I-*Eco*RI fragment from pTZ-KE and the 0.67 kb *Eco*RI-*Hind*III fragment from pTZ-EB behind a poly-histidine tag into *Kpn*I and *Hind*III sites of the plasmid vector RsetB to create RsetB-90. This construct was used to transform competent BL21 cells**.**

Recombinant BL21 cells expressing His-TAA90K were grown at 30 ^{o}C in Luria broth to an optical density of 0.6-0.8 at 600 nm and induced for 4 hours with 1 mM isopropylthiogalactoside (IPTG) (Invitrogen, Burlington, ON). Bacteria were pelleted by centrifugation at 1500 x g and lysed by rocking for 30 minutes in buffer containing 50 mM Tris HCl pH 7.4, 0.1% Triton X-100, 0.25 mM phenylmethylsulfonylfluoride (PMSF), and 0.03 mg/mL lysozyme. The sample was sonicated (1 x 15 sec bursts at 350 watts) and centrifuged at 14,000 rpm in a Beckman microcentrifuge, in preparation of metal chelation affinity chromatography (see below).

4.3 Preparation of recombinant vaccinia virus expressing TAA90K-His

For expression of TAA90K in eukaryotic cells, recombinant vaccinia virus encoding TAA90K containing a C-terminal poly-histidine tail was constructed. To this end, a histidine-tag consisting of 6 consecutive histidine residues was attached to the 3' end of the TAA90K open reading frame (Laferté *et al*., 2000), as follows. First, using the Quikchange mutagenesis kit (Stratagene, La Jolla, CA) and the mutagenesis primers Mac2.SalU (CTCCTCAGGTGT**C**GAC*TAG*ACGGCGTGG) and Mac2.SalL (CCACGCCGTCTAGTC**G**ACACCTGAGGAG), a unique *Sal*I site (underlined) was created at the 3' end of the TAA90K ORF just before the stop codon TAG (in italics) without altering its predicted amino acid sequence. The mutated nucleotide in the mutagenesis primer is shown in bold. Then, the 0.62 kb *Eco*RI-*Sal*I fragment representing the mutated 3' end of the TAA90K ORF was inserted between the *Eco*RI and *Xho*I sites of the plasmid vector pCITE-2a to create pCITE-EB90His, thereby placing the coding sequence for the 6-histidine residues in frame with the 3' end of the TAA90K ORF. However, the destruction of the *Sal*I site in the cloning process also resulted in a D to E conversion in the C-terminal amino acid residue of TAA90K.

The TAA90K ORF containing a C-terminal histidine tag (TAA90K-His) was reconstituted by replacing the 0.62 kb *Eco*RI-*Xba*I fragment of pCD90 (Laferté *et al.,* 2000) with the 0.62 kb *Eco*RI-*Xba*I fragment from pCITE-EB90His to create pCD TAA90His. Similarly, the TAA90K-His ORF was introduced into the pSLAVE vector (developed at Vaccine and Infectious Disease Organization) by replacing the *Eco*RI-*Bgl*II fragment of pSL- TAA90K with the 0.63 kb *EcoR*I-*Bgl*II fragment from pCITE-

EB90His to create pSL-TAA90K-His. In this way, the TAA90K-His ORF was inserted behind a synthetic vaccinia late promoter and a site in the vector flanked by coding regions of the wild-type vaccinia virus thymidine kinase (TK) gene.

Recombinant vaccinia virus encoding TAA90K-His was constructed by disrupting the TK gene in wild-type vaccinia virus with the TAA90K-His coding sequence via homologous recombination. CV-1 cells were infected with wild-type vaccinia virus and transfected with linearized pSL-TAA90K-His DNA by electroporation (Gene Pulser, BioRad, Mississauga, ON). Recombinant vaccinia virus was selected in Rat-2 cells (TK^-) in the presence of 5-bromodeoxyuridine (BUdR) and isolated by plaque purification, as described previously (Loh *et al*., 1994). Similarly, recombinant vaccinia virus encoding wild-type TAA90K, without a histidine tag, was generated by inserting the entire open reading frame for TAA90K into the pSLAVE vector to create pSL-TAA90K.

Transfection of plasmids encoding wild-type or His-tagged TAA90K (i.e. pCD90 or pCDTAA90K-His) into COS-1 cells (15 μg DNA/2 X 10^6 cells) was carried out by electroporation with the Gene Pulser apparatus (BioRad, Mississauga, ON) as previously described (Chu *et al*., 1987). Electroporation was carried out using a 0.4 cm cuvette at a voltage setting of 250 V and a capacitance setting of 500 μF.

4.4 Infection of cells with recombinant vaccinia virus expressing TAA90K or TAA90K-His

HT-29 cells grown to 70% confluency were cultured in complete medium containing 2% FBS and infected with recombinant vaccinia encoding TAA90K-His/TAA90K at a multiplicity of infection (m.o.i) of 0.01. At 24 hours post-infection, cells were washed twice in PBS and cultured in complete F12/DMEM medium. At 48 and 72 hours post-infection, conditioned medium was collected, centrifuged at 3000 rpm in a Sorvall bench top centrifuge to remove cells. The supernatant was centrifuged for 4 hours at 16,300 x g to pellet virus. The resulting supernatant was filtered through a 0.2 μm filter (Millipore Corporation, Bedford, MA), dialyzed against three changes of TBS pH 7.4 (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.02% sodium azide (NaN_3))

adjusted to 0.5 M NaCl and subjected to affinity chromatography on wheat germ agglutinin (WGA)-agarose, as described below.

In some experiments, HT-29 cells were treated with the glycosylation inhibitors 1-deoxymannojirimycin (DMJ) (Sigma, Oakville, ON) or swainsonine (Sw) (Sigma, Oakville, ON), at concentrations of 0.3 mM and 5.8 μM respectively, approximately 6 h after plating. In these experiments, the inhibitors DMJ and Sw resulted in the generation of TAA90K glycoforms referred to as TAA90K-DMJ or TAA90K-Sw, respectively.

4.5 Metal chelation chromatography of histidine-tagged proteins

The bacterial pellet (from section 4.2) consisting of inclusion bodies containing His-TAA90K was solubilized in 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl and 5 mM imidazole (binding buffer) containing 1 mM PMSF and 6 M guanidine-HCl. Following centrifugation, the solubilized samples were applied to 0.5 mL of His-bind resin charged with Ni^{2+} ions (Novagen, Madison, WI) pre-equilibrated in binding buffer containing 6 M guanidine-HCl. The His-bind resins were washed with 20 column volumes of binding buffer and wash buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 60 mM imidazole) each containing 6 M urea. His-TAA90K was eluted with 10 column volumes of elution buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 M imidazole) containing 6 M urea. Fractions containing recombinant proteins were identified employing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis. The urea concentration of the pooled fractions containing bacterially expressed His-TAA90K was reduced in a stepwise manner by dialysis against solutions of TBS (pH 7.4) buffer containing decreasing concentrations of urea, down to a final concentration of 2 M urea.

A similar protocol was utilized for the isolation of TAA90K-His from the conditioned medium of HT-29 cells infected with recombinant virus. Modifications included the use of a 5 mL His-bind resin column and buffers lacking guanidine-HCl or urea.

4.6 Monoclonal and polyclonal antibodies

TAA90K-specific monoclonal antibodies were prepared as described previously (Laferté and Loh, 1992; Laferté *et al*., 2000). IgG fractions were purified from mouse ascites by affinity chromatography on protein G-agarose according to manufacturer's instructions (Amersham Biosciences, Baie D'Urfé, QC). Polyclonal antiserum specific for bacterially-expressed His-TAA90K was prepared in rabbits following an injection of 50 μg of bacterially expressed His-TAA90K followed by two boosts with the recombinant protein at 4 week intervals, following protocols approved by the Canadian Council on Animal Care. Monoclonal antibodies to pro- and active MMP-7 were purchased from Chemicon (Temecula, CA) and the Penta-His antibody specific for the histidine tag was purchased from Qiagen (Mississauga, ON).

4.7 Lectin-affinity chromatography

Conditioned medium containing TAA90K, TAA90K-His, TAA90K-DMJ or TAA90K-Sw was dialyzed against three changes of TBS pH 7.4 (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.02% NaN_3), adjusted to 0.5 M NaCl and applied to a 5 mL column of wheat-germ agglutinin (WGA)-agarose (E-Y Labs, San Mateo, CA) pre-equilibrated in 50 mM Tris-HCl pH 7.4, 0.5 M NaCl, 0.02 % NaN_3 (WGA buffer). After washing the column with 10 volumes of WGA buffer, TAA90K, TAA90K-His or TAA90K-Sw were eluted with TBS (pH 7.4) containing 5 % N-acetylglucosamine (Sigma, Oakville, ON). Fractions containing the different forms of TAA90K, as determined by dot blot analysis employing the TAA90K-specific monoclonal antibody 1H9 (Laferté and Loh, 1992) and SDS-PAGE, were pooled and dialyzed against 50 mM Tris-HCl (pH 7.0).

4.8 Ion-exchange chromatography

WGA-agarose eluted fractions containing TAA90K/TAA90K-His or TAA90K-Sw or the WGA-agarose flow through fraction containing TAA90K-DMJ were dialyzed against 50 mM Tris-HCl (pH 7.0), and applied to a 1 mL column of cation-exchange resin (HiTrap SP HP, Amersham Biosciences, Baie D'Urfé, QC), equilibrated in the same buffer. The eluted fractions containing TAA90K-DMJ or the flow-through

fractions containing TAA90K/TAA90K-His or TAA90K-Sw were adjusted to 0.2 M NaCl and purified further on a 1 mL column of anion-exchange resin (HiTrap Q HP, Amersham Biosciences, Baie D'Urfé, QC) equilibrated in 50 mM Tris-HCl (pH 7.0), 0.2 M NaCl. After washing, the anion-exchange column was connected to a fast performance liquid chromatography system (FPLC, Amersham Biosciences, Baie D'Urfé, QC), and TAA90K was eluted with a linear gradient of 0.2 to 1M NaCl. The absorbance profile at 280 nm for the purification of TAA90K/TAA90K-His revealed a single peak eluting at 0.6 M NaCl which consisted of TAA90K/TAA90K-His, as detected by SDS-PAGE and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (Proteomics Laboratory, Plant Biotechnology Institute, Saskatoon, SK). Similarly, TAA90K-Sw eluted in a single peak at 0.6 M NaCl, however, TAA90K-DMJ bound more tightly to the anion-exchange column, eluting between 0.65 M and 0.7 M NaCl.

Fractions containing TAA90K-His/TAA90K, TAA90K-DMJ or TAA90K-Sw were dialyzed against three changes of 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl and concentrated by re-application of the sample to the anion-exchange column and elution in a minimum volume of column buffer containing 1 M NaCl. The fractions containing TAA90K were desalted on a NAP-5 column (Amersham Biosciences, Baie D-Urfé, Qc). Typical yields of TAA90K isolated in the absence of glycosylation inhibitors ranged from 300-500 μg/L of conditioned medium.

4.9 Proteolytic cleavage of TAA90K by MMPs

ProMMP-2 and proMMP-9 (Calbiochem, La Jolla, CA) were converted to their active forms following incubation with 1 mM p-aminophenylmercuric acetate (APMA) in dimethylsulfoxide (DMSO) at 37 ^{o}C for 24 hours. Activated MMP-2, MMP-9 or active MMP-7 (Calbiochem, La Jolla, CA) were each incubated with TAA90K at a ratio of 1:3 (w/w) respectively, in cleavage buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 10 mM calcium chloride ($CaCl_2$), 5 μM zinc sulfate ($ZnSO_4$), 0.05% Brij-35 (polyoxyethylene lauryl ether) and 0.02% NaN_3 for 1 h at 37 ^{o}C. In order to inhibit metalloproteinase enzyme activity, samples were incubated in buffer consisting of 50 mM Tris-HCl (pH 7.5), containing 0.2 M NaCl, 50 mM ethylenediaminetetraacetic acid

(EDTA), 0.05% Brij-35 and 0.02% NaN_3. Inhibition of enzyme activity was also achieved by boiling the enzyme for 4 minutes in cleavage buffer prior to incubation with TAA90K.

4.9. Purification of TAA90K proteolytic fragments for use in biological assays

MMP-7 and TAA90K combined at a ratio of 1:50 (w/w), respectively, were incubated in cleavage buffer for 24 h at 37 ^{o}C. The digestion mixture was diluted 20-fold with TBS pH 7.4 to lower the concentration of detergent, salt and divalent cations prior to anion-exchange chromatography on a 1mL HiTrap Q HP column equilibrated in TBS pH 7.4. While the flow-through fraction contained the MMP-7, proteolytic fragments of TAA90K were eluted from the column with 50 mM Tris-HCl pH 7.0 containing 1 M NaCl. Fractions containing the TAA90K fragments were desalted on a NAP-5 column.

4.10 Amino acid sequencing of proteolytic fragments of TAA90K cleaved by MMP-7

An aliquot containing 4 μg of cleaved TAA90K was resolved on a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue. After destaining the gel, the 79 kDa and 22 kDa bands were excised, washed several times with HPLC-grade water and submitted to the Harvard Microchemistry and Proteomics Analysis Facility (Harvard University, Boston, MA) for N-terminal sequencing. The primary internal cleavage site at Asp-452 of the TAA90K protein was confirmed by C-terminal sequencing and mass spectrometry analysis of trypsin- and endopeptidase LysC-digests of the N-terminal 79 kDa fragment. To this end, cleaved TAA90K was separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Bedford, MA). After staining of the membrane for 1 min in a solution of 0.1% Ponceau Red in 1% acetic acid, the 79 kDa band was excised, washed twice in 50% acetonitrile and subjected to proteolytic digestion. The resulting peptides were analyzed by microcapillary reverse-phase HPLC nano-electrospray mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer.

4.11 Detection of TAA90K and MMPs in conditioned medium

Cells grown to 70% confluency were cultured in complete F12/DMEM medium. In some experiments, TAA90K (1 μg or 5 μg) was added to HT29 cells for 24 h, 48 h or 72 h. Conditioned medium from colon cancer cell lines, including HT29 cells, grown in the presence of absence or TAA90K was collected and centrifuged at 3000 rpm in a Sorvall bench top centrifuge to pellet cells. The medium was concentrated five-fold using a Centricon centrifugal filter device (Millipore Corporation, Bedford, MA) and subjected to ELISA analysis using the TAA90K-specific monoclonal antibody 1H9, mouse anti-proMMP-7 or mouse anti-MMP-7 antibodies (Chemicon, Temecula, CA).

4.12 Metabolic labeling of cells

Cells were radiolabeled for 24 h in methionine-free DMEM supplemented with one-tenth the normal content of methionine, 10% FCS and 50 μCi/mL of [S^{35}]-methionine/mL (800 Ci/mmol; NEN, Calgary, Alberta, Canada) as described previously (Laferté and Loh, 1992). The medium was collected and centrifuged at 3000 rpm in a Sorvall desk top centrifuge to pellet any cells.

4.13 Preparation of tissue lysates

Samples from human colon tumors and adjacent normal colon (400-500 mg; Asterand, Inc., Detroit, MI) were homogenized using a Polytron homogenizer (Brinkmann Instruments, Edmonton, AB) in 8 volumes of 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM PMSF, 1 mM benzamidine, 10 μg/mL aprotinin, 0.5 μM leupeptin and 0.7 μM pepstatin. The sample was adjusted to 1% Triton X-100, incubated on ice for 1 h and centrifuged at 20, 000 *g* for 30 min. The resulting supernatant was assayed for protein content and analyzed by SDS-PAGE and Western blotting.

4.14 SDS-Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Briefly, proteins were separated on a 7.5% or 10% polyacrylamide gel for 1-1.5 hrs at 100 volts using a Mini-Protean II electrophoresis system (BioRad Laboratories, Mississauga, ON). Proteins were

visualized by staining with 0.088% Coomassie Blue R-250 (BioRad Laboratories, Mississauga, ON) in 40% ethanol, 8% acetic acid for 30 minutes followed by destaining in a solution containing 10% acetic acid and 20% methanol. [^{35} S]-methionine labeled proteins were separated by SDS-PAGE and visualized by fluorography after enhancement with Entensify (NEN, Perkin Elmer, Boston, MA).

4.15 Dot blot analysis

Dot blot analysis was performed as originally described in Sharon *et al.* (1979). Aliquots of 10 μL were spotted directly onto nitrocellulose (Schleicher and Schull, Mandel Scientific, Guelph, ON) and allowed to dry at room temperature followed by blocking overnight at 4 ^{o}C in TBS containing 4% BSA. The nitrocellulose was washed three times for 5 minutes with TBST (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% BSA, 0.025% Tween, 0.02% NaN_3), incubated at room temperature for 2 hours with the primary antibody diluted in TBST, washed three times with TBST and incubated for 1 hour with alkaline-phosphatase-conjugated affinity-purified goat anti-mouse or goat anti-rabbit Ig (BioRad Laboratories, Mississauga, ON) diluted 1:3000 in TBST. The nitrocellulose was washed three times with TBST, once with TBS (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.02 % NaN_3) containing 0.05 % Tween (BioRad Laboratories, Mississauga, ON), once with TBS and once with developing buffer (0.1M Tris-HCl, pH 9.5, 0.1M NaCl, 50 mM $MgCl_2$). Immunoreactivity was detected colourimetrically by incubating the nitrocellulose in developing buffer containing 3.67 μM p-nitroblue tetrazidium chloride (NBT) (BioRad Laboratories, Mississauga, ON) and 3.46 μM 5-bromo-4-chloro-3-indolyl-phosphate-toluidine salt (BCIP) (BioRad Laboratories, Mississauga, ON) at room temperature.

4.16 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISA) were performed as described in Laferté *et al.* (1995). Samples were applied in a final volume of 100 μL to the wells of a Nunc-Immuno 96-well plate with a PolySorb surface (VWR International, Edmonton, AB) overnight at 4 ^{o}C. After washing with TBS pH 8.0, wells were blocked with 200 μL TBS containing 4% BSA for 1h at room temperature. Wells were washed three

times with TBST followed by incubation for 2 hours at room temperature with 100 μL primary antibody diluted in TBST. After washing the wells with TBST, plates were incubated for 1 hour at room temperature with 100 μL alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit Ig (BioRad Laboratories, Mississauga, ON) diluted 1:3000 in TBST. Wells were washed three times with TBST, once with TBS containing 0.05% Tween-20 and once with TBS pH 8.0. Immunoreactivity was detected colorimetrically at 405 nm following addition of 100 μL p-nitrophenyl phosphate substrate per well (0.5 mg/mL in 10 mM diethanolamine buffer) (BioRad EIA kit, BioRad Laboratories, Mississauga, ON).

4.17 Indirect immunofluorescence analysis

Indirect immunofluorescence analysis of live cells was performed as described in Laferté and Loh (1992) with the following modifications. Briefly, 2 x 10^5 HT29 cells in 3% FCS/PBS were pre-incubated with either 10 μg BSA or 10 μg galectin-3 for 1 h on ice, prior to incubation with either 10 μg TAA90K or 10 μg BSA for 1 h on ice. Following washes, cells were incubated with either normal mouse IgG or MAb 1H9 (1:200) for 1 h. After washing, cells were incubated for 30 min with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse Ig (1:50) (Dimension Laboratories, Mississauga, Ontario, Canada). Cells were dropped onto glass slides and mounted in PBS/glycerol (1:1 v/v).

For analysis of fixed and permeabilized cells, cells were harvested, washed in PBS and applied to toxoplasmosis slides (Bellco Glass, Inc., Vineland, NJ, USA). After drying, slides were submerged in -20^{o}C methanol for 4 minutes followed by submersion in -20^{o}C acetone for two minutes. After drying, slides were washed in PBS and incubated with primary antibody diluted 1:250 in 3% BSA/PBS for 1 h. After washing, slides were incubated for 30 min with FITC goat-anti mouse Ig (1:50) and washed. Coverslips were applied using PBS/glycerol (1:1 v/v). Fluorescence of both fixed and live cells was visualized employing a Nikon epifluorescence microscope.

4.18 Western blotting analysis

Proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose membranes (Schleicher and Schüll, Mandel Scientific, Guelph, ON) for 2 hours at 100 volts (Tobwin *et al*., 1979). Nitrocellulose blots were blocked overnight at 4 ^{o}C in TBS (pH 8.0)/4% BSA. Following three 5-min washes in TBST buffer (TBS pH 8.0, 0.1% BSA, 0.025% Tween-20), blots were incubated for 2 hours with primary antibody. Following three 5-minute washes in TBST, blots were incubated for 1 hour with alkaline phosphatase-labeled, affinity purified goat anti-rabbit Ig or anti-mouse Ig (Bio-Rad Laboratories, Mississauga, ON). The substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) were used for color development.

4.19 Immunoprecipitation analysis

Crude cell lysates (prepared as described in section 4.13) or media collected from cells grown in culture were incubated with primary antibodies overnight at 4 ^{o}C. Protein A-Sepharose CL-4B (Amersham Biosciences, Baie D'Urfé, QC) was resuspended in TBS pH 8.0, 1% Nonidet P-40 (BDH), 1% deoxycholate, 1 mM benzamidine, 0.02 % NaN_3(IP buffer) to a final concentration of 80 mg/mL. Samples were incubated with 100 μL of Protein A suspension on a nutator (Becton, Dickinson and Company, Sparks, MD) for 1 hour at 4^0C. After centrifugation, Protein A-Sepharose beads were washed once with IP buffer, once with IP buffer containing 0.5 M sodium chloride (VWR International, Edmonton, AB), once with IP buffer containing 0.1% SDS (BioRad Laboratories) followed by a final wash in IP buffer. Protein A Sepharose beads were boiled in SDS-PAGE sample buffer (12.5 mM Tris-HCl pH 6.8, 2 % 2-mercaptoethanol, 2% SDS, 10 % glycerol and bromophenol blue (BioRad Laboratories, Mississauga, ON) and the supernatants resolved on SDS-PAGE gels.

4.20 Immunohistochemical staining of normal and neoplastic tissues

Sections of human colon tumors from 47 archived paraffin specimens collected at Royal University Hospital (Saskatoon, SK) as well as normal colon from five individuals who died of causes unrelated to cancer (Asterand, Inc., Detroit, MI) were

stained with TAA90K-specific monoclonal antibody 4D1 (Laferté *et al.,* 2000). Five micron-thick tumor sections were deparaffinized in xylene and prepared for immunohistochemical staining with the Envision^{TM} plus system mouse staining kit (Dako, Mississauga, ON), according to manufacturer's instructions with the following modifications. Tumor sections immersed in PBS were heated in a microwave oven for 20 minutes (4 x 5 min), cooled and washed in EDTA buffer at pH 9.0 prior to blocking of endogenous peroxidase. After washing in PBS, sections were incubated for 2 h with either normal mouse IgG (Sigma, Oakville, ON) or 4D1 IgG (5 μg/mL in 1% bovine serum albumin). After chromogen treatment and washing, sections were dehydrated and mounted in Entellan (VWR, Edmonton, AB). A section of human colon tumor previously shown to stain strongly with MAb 4D1 was included in each experiment as an internal control. To confirm the specificity of MAb 4D1, blocking studies were carried out by pre-incubating tissue sections overnight with 70 μg/mL purified TAA90K-His. Stained sections were assessed independently by two pathologists using a Nikon Eclipse 80i microscope equipped with a digital imaging system and scored as positive if at least 10% of the cells within the tumor stained with MAb 4D1 but not with normal mouse IgG.

4.21 Determination of protein concentration

The concentration of protein in samples was determined using the BioRad DC protein assay kit (BioRad Laboratories, Mississauga, ON) following the manufacturer's instructions. Briefly, dilutions of the sample in a final volume of 50 μL were incubated at room temperature for 15 min with 250 μL alkaline copper tartrate solution (reagent A) and 2 mL of Folin reagent (reagent B). Reagent A was supplemented with 20 μL reagent S per mL of reagent A when detergent was present in the samples. The absorbance of the samples was measured at 750 nm and compared to a standard curve of gamma-globulin ranging from 0.2 to 1 mg/mL.

4.22 Solid-phase binding assay

The ability of TAA90K to bind to galectins, extracellular matrix proteins and MMPs was tested using a solid-phase assay. Briefly, 0.5 μg of either galectin-1, galectin

-3, collagen I, collagen IV, fibronectin, laminin-1 (Sigma, Oakville, ON), laminin-5, laminin -10 (Chemicon, Temecula, CA) or the pro- or active forms of MMPs -1, -2, -7 -9 (Calbiochem, La Jolla, CA) or BSA (Sigma, Oakville, ON) were added, in triplicate, to PolySorb 96-well plates and allowed to adsorb to the plastic surface overnight at 4 ^{o}C. After washing 3 times in PBS (pH 7.0), residual protein-binding sites in the wells were blocked with PBS/5% BSA (blocking buffer) for 2 hours at room temperature. Following 5 washes with PBS/0.04% Tween (PBST), TAA90K or BSA (2 μg/well) diluted in blocking buffer was added to each well and incubated overnight at 4 ^{o}C. The wells were washed 5 times with PBST and incubated for 2 hours at room temperature with TAA90K-specific monoclonal antibody 1H9 (1:1000 dilution in blocking buffer). Following 5 washes in PBST, wells were incubated with alkaline phosphatase-labeled, affinity-purified goat anti-mouse Ig (1:3000 dilution in blocking buffer) (Bio-Rad Laboratories, Mississauga, ON). Bound TAA90K was detected colorimetrically at 405 nm following addition of the substrate p-nitrophenyl phosphate (BioRad EIA kit, BioRad Laboratories, Mississauga, ON). To examine the effect of inhibitors of carbohydrate-dependent binding on the TAA90K/galectin-3 interaction, similar experiments were carried out except that wells were pre-incubated for 1 hour with 500 mM lactose or 150 mM lactose containing 2 mg/mL asialofetuin (2X concentration) before addition of an equal volume of buffer containing TAA90K (Kopitz *et al.,* 2001).

4.23 Cellular adhesion assay

Cell adhesion to TAA90K, galectin-3, extracellular matrix proteins or BSA was measured using a colorimetric assay (Takada *et al.,* 1988). Wells of PolySorb 96 well plates were coated overnight at 4 ^{o}C with serially diluted samples of extracellular matrix proteins, TAA90K, galectin-3 or BSA. Prior to use, commercially available galectin-3 (Calbiochem, La Jolla, CA) was subjected to chromatography on a NAP-5 column (Amersham Biosciences, Baie D'Urfé, QC) to remove lactose. Following 3 washes with PBS, wells were blocked with PBS/4% BSA for 1 hour at room temperature. After 5 washes with PBS, wells were incubated with 0.2 mL DMEM supplemented with 32 μg/mL gentamycin containing 10^5 cells for 4 hours at 37 ^{o}C in a 5% CO_2 incubator. Non-adherent cells were removed by washing with PBS until cells were no longer

detected in the BSA-coated wells. Attached cells were fixed for 30 minutes with PBS/4% formaldehyde, washed 3 times with PBS and stained for 15 minutes with 0.05 mL of a 1% toluidine blue solution. Following 3 washes in PBS, the dye was extracted with 200 μL methanol and the optical density was measured at 620 nm. In some experiments, HT29 cells were pre-treated for 48 hours with the glycosylation inhibitors deoxymannojirimicin (0.3 mM) or swainsonine (5.8 μM). In other experiments, the ability of TAA90K to inhibit galectin-3-mediated cell binding was examined. In this case, TAA90K diluted in DMEM supplemented with gentamycin was added to wells previously coated with galectin-3 and incubated for 1 hour at 37 ^{o}C prior to addition of cells.

4.24 Cellular migration assay

Migration assays were performed employing transwell chambers containing polycarbonate inserts with 8 μm pores (Costar, Corning, Fisher Scientific, Nepean, ON) as described (Jasiulious *et al.,* 1996) with the following modifications. The underside of the filter was incubated for 2 h at 37 ^{o}C with solutions of extracellular proteins including fibronectin, collagen I, laminin-1 (Sigma, Oakville, ON), galectin-3 (Calbiochem) or TAA90K diluted to 50 μg/mL with DMEM. After washing with PBS, filters were blocked with PBS containing 0.5% BSA for 1 hour at 37 ^{o}C. Following three washes with PBS, filters were placed in a 24-well cluster dish (Costar, Corning, Fisher Scientific, Nepean, ON) containing 600 μL complete F12/DMEM media with or without 5 μg TAA90K. One hundred microliters of F12/DMEM media containing 2 x 10^5 cells was added to the top of the filter and cells were allowed to migrate to the underside of the filter at 37 ^{o}C for 48 hours. In some experiments, 5 μg TAA90K was added with the cells to the top of the filter. In order to assess the extent of cellular migration, 20 μL of a 5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide: thiazolyl blue, Sigma, Oakville, ON) solution was added to the bottom chamber of the transwell plates and incubated for 4 hours at 37^{o}C. Non-migrating cells that remained in the upper chamber had not migrated and were removed with a cotton swab. Cells that had migrated through the pores and adhered to the

underside of the membrane were extracted with DMSO and the optical density was read at 540 nm.

4.25 Cellular invasion assay

Experiments assessing cellular invasion were performed employing BD BioCoat Matrigel Invasion Chambers (Fisher Scientific, Nepean, ON) according to manufacturer's instructions with some modifications. The invasion chambers consisted of 24-well plates containing 12 polycarbonate inserts with 8 μm pores coated with reconstituted extracellular matrix (Matrigel). After the Matrigel was rehydrated in warm DMEM for 2 h at 37°C, some wells were pre-incubated with increasing amounts of TAA90K and/or galectin-3 for 1h at 37°C before 2.5 x 10^4 cells were added to the inserts. In some experiments, increasing amounts of TAA90K and/or galectin-3 were added with the cells to the inserts. DMEM or DMEM complete medium containing 10% FCS was added to the lower chambers of the plate. Invasion chambers were incubated for 24-48 h at 37°C. After non-invading cells were removed from the upper surface of the inserts with a cotton swab, invading cells found on the underside of the filter and in the lower chamber were stained with MTT as described in the previous section.

4.26 Statistical analysis

In the case of protein binding, ELISA analysis and cellular assays, sample means were compared using the two-tailed student's *t* test. Statistical analysis was performed using Microsoft Excel. *P* values < 0.05 were considered statistically significant.

5.0 RESULTS:

5.1 EXPRESSION OF TAA90K IN COLON CANCER

5.1.1 Immunohistochemical staining of colon tumors and normal colonic mucosa

In order to examine the expression pattern of TAA90K in human colon cancer, we carried out immunohistochemical analysis of paraffin-embedded colon tumors using 4D1, a TAA90K-specific monoclonal antibody previously developed in our laboratory (Laferté *et al*., 2000). We examined 47 colon tumors of varying histopathological grade including 10 Duke's A, 5 Duke's B, 31 Duke's C and 1 Duke's D tumor. In contrast to normal colon which lacked detectable TAA90K expression (Figure 5.1, panel A), elevated expression of TAA90K was detected in all colon tumors examined. There were a number of staining patterns observed for TAA90K expression in colon tumor sections, including diffuse intracellular staining (panel C), granular staining at the apical border of colon cancer cells (panel D) and punctate staining consistent with proteins associated with secretory vesicles (panel E), staining of luminal secretions (panel F) and in some cases, basolateral staining (panel G). Staining of the transitional mucosa at the tumor border is shown in panel H. The intensity of stained sections varied from weak to intense and the percentage of positively stained cells within the tumor ranged from 10% to more than 50%. Immunohistochemistry carried out in the presence of purified TAA90K effectively blocked the complex staining pattern that was previously observed (panel B), confirming that the staining pattern was due to TAA90K expression.

5.1.2 Western blotting analysis of colon tumors and normal colonic mucosa

In order to confirm the elevated expression of TAA90K in colon tumors compared to normal colon, we immunoprecipitated TAA90K from tissue lysates of three colon tumors (Duke's B, C and D) and adjacent normal colon using monoclonal antibody 1H9. Immunoprecipitates were analyzed by Western blotting using a polyclonal antibody raised against a bacterially expressed recombinant TAA90K

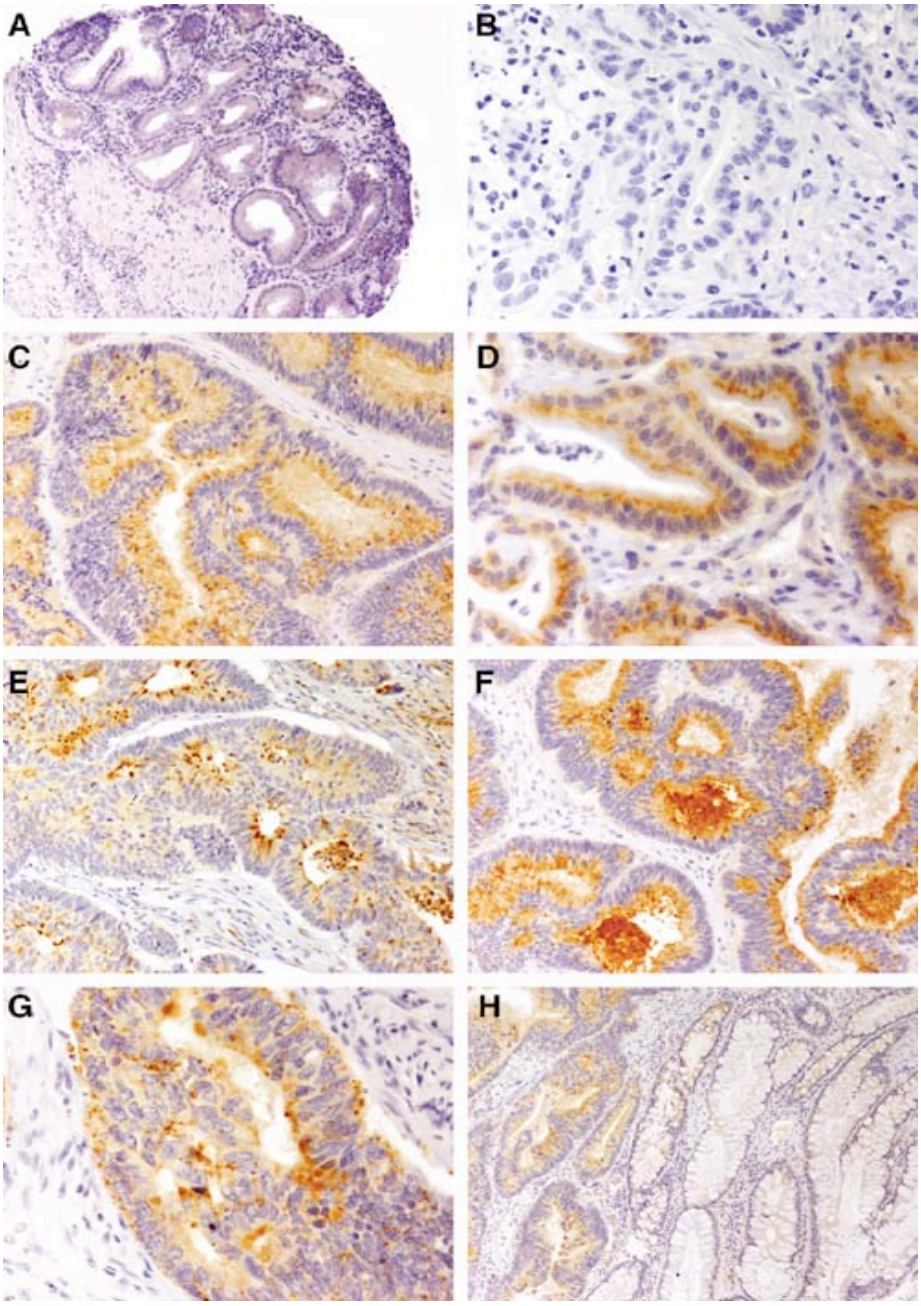


Figure 5.1: Immunohistochemical staining of human colon tumors. Sections of human colon tumors were examined by immunohistochemical staining with MAb 4D1 (5 μg/ml) or normal mouse IgG. Staining patterns obtained with normal IgG are not shown since they yielded negative results. (A) Normal colon, 400×, (B) Duke's B2 tumor (also shown in panel D) incubated with MAb 4D1 pre-absorbed with TAA90K-His (70 μg/ml), (C) Duke's C2 tumor showing diffuse intracellular staining, 200×, (D) Duke's B2 tumor showing granular staining at the apical border of colon cancer cells, 400×, (E) Duke's C1 tumor showing punctate staining consistent with secretory vesicles, 400×, (F) Duke's C2 tumor showing luminal staining, 400×, (G) Duke's A tumor showing apical and basolateral staining, 400×, and (H) Duke's C2 tumor showing staining of transitional mucosa at the tumor border, 200×.

containing an N-terminal poly-His tag (His-TAA90K). As shown in Figure 5.2, colon tumors expressed higher amounts of TAA90K than normal colonic mucosa. The apparent mobility of TAA90K in the Duke's B, C and D samples was 106, 101 and 108 kDa respectively, consistent with variable glycosylation of TAA90K previously detected in human colon carcinoma cell lines (Laferté and Loh, 1992). The lower molecular mass species of 76-86 kDa detected in each tumor sample likely results from proteolytic cleavage of TAA90K previously detected in various cell lines (Laferté and Loh, 1992; Koths *et al.,* 1993; Sasaki *et al*., 1998) These results suggested that TAA90K may represent a novel tumor-associated marker for colon cancer.

5.1.3 Secretion of TAA90K by human colon carcinoma cell lines

In addition to examining the expression of TAA90K by human colon carcinoma tissues, we also examined the secretion of TAA90K by a number of human colon cancer cell lines including Caco-2, LoVo, LS123, HT-29, KM20C, SW403 and SW620. These cell lines were chosen because they were derived from either primary colon tumors of varying grade or from metastases.

Cell line	**Source**
Caco-2	Primary colon tumor
LoVo	Metastatic nodule in the supraclavicular region
LS123	Primary colon tumor, Duke's B
HT-29	Moderately differentiated grade II
KM20C	Liver metastasis
SW403	Grade III adenocarcinoma
SW620	Lymph node metastasis

Conditioned medium from the cell lines was analyzed for the secretion of TAA90K by ELISA employing MAb 1H9 which is specific for TAA90K (Figure 5.3). Interestingly,

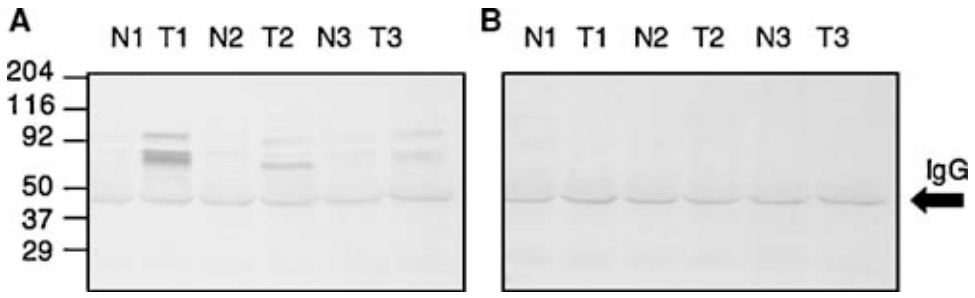


Figure 5.2: Analysis of TAA90K expression in human colon tumors and adjacent normal colonic mucosa. TAA90K was immunoprecipitated from 0.5 mg of detergent-solubilized human colon tumors (T1 to T3) or adjacent normal colonic mucosa (N1 to N3) using 5 μl of either MAb 1H9 (panel A) or NMS (panel B). Immunoprecipitates were analyzed by Western blotting using rabbit anti-TAA90K antiserum (1:2,000 dilution). The samples designated T1 to T3 and N1 to N3 correspond to Duke's B, Duke's C and Duke's D tumors and their adjacent normal colonic mucosa, respectively. The size of the molecular weight markers in kilodaltons is shown at the left of the figure. The mobility of the immunoglobulin band (IgG) is shown at the right of the figure.

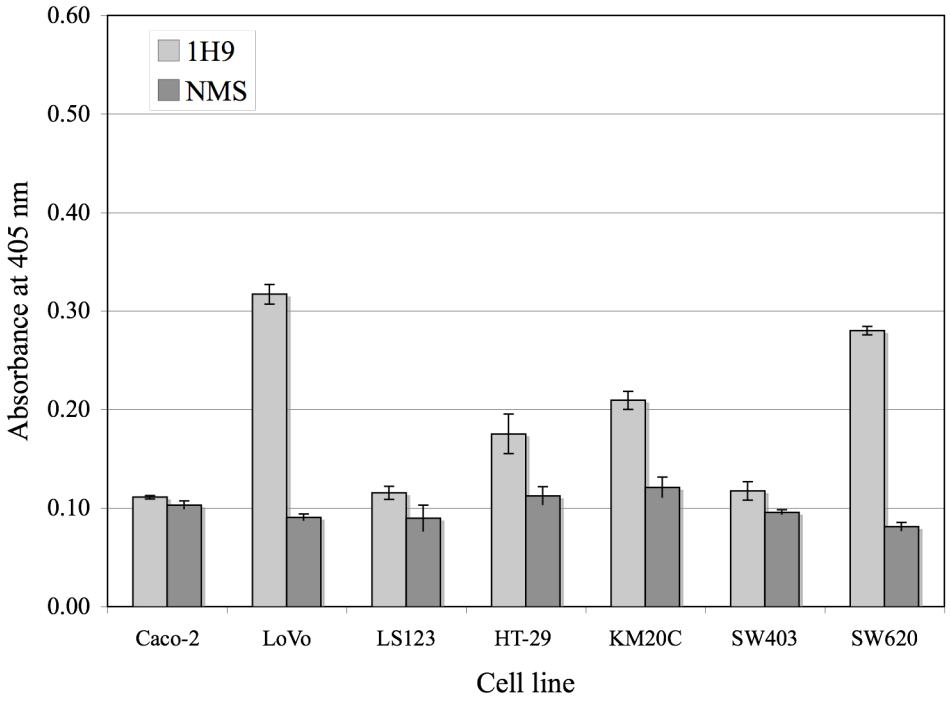


Figure 5.3 TAA90K secretion by human colon cancer cell lines. The conditioned serum-free medium from colon cancer cell lines was concentrated 5-fold and coated (100μL) in triplicate in a 96 well plate. Wells were washed, blocked with TBS containing 4% BSA, washed and incubated with MAb 1H9 or NMS control (1:1000) for 2 h at room temperature. After washing, wells were incubated with alkaline phosphatase-conjugated goat anti-mouse Ig (1:3000) for 1h followed by washing and addition of colorimetric substrates. The absorbance was read at 405 nm. The mean and standard deviation of triplicate samples are shown.

the cell lines that express the highest levels of TAA90K are LoVo, SW620 and KM20C, which were all isolated from metastases.

5.2 OPTIMIZATION OF TAA90K ISOLATION FROM HUMAN COLON CANCER CELL LINES

Immunohistochemical analysis revealed increased extracellular accumulation of TAA90K in colon tumors compared to normal colon suggesting that TAA90K may play a role in colon cancer progression by affecting cell-cell or cell-extracellular matrix (ECM) interactions. Analysis of the conditioned medium of a panel of human colon cancer cell lines indicated that secretion of TAA90K is maintained in these established cell lines, providing a model system to study TAA90K function and a source of mature, fully glycosylated TAA90K. Thus, in order to identify the possible mechanism(s) of TAA90K function in colon cancer, we purified TAA90K from the conditioned medium of human colon carcinoma cell line HT-29 in sufficient quantity to carry out functional studies. The HT-29 cell line was selected as a source of TAA90K because it was established from a primary colon tumor, it maintains a typical epithelial morphology, it grows to high density and secretes moderate levels of TAA90K.

5.2.1 Purification of TAA90K from human cell lines

Initially, attempts were made to purify TAA90K directly from HT-29 conditioned medium. Ammonium sulfate precipitation of the conditioned medium followed by dialysis was used as a means to concentrate the TAA90K sample and remove unwanted proteins. This approach was not pursued further because of reduced solubility of the TAA90K preparation following dialysis (data not shown). As an alternative, the conditioned medium was subjected to lectin affinity chromatography on WGA-agarose, a useful method for purifying glycoproteins modified by N-acetylglucosamine and/or sialic acid (Peters *et al*., 1979). Although this approach was successful in enriching for TAA90K, an additional gel exclusion chromatography step on Superose 6, followed by sample concentration using an Amicon centrifugation device, were required to remove contaminating proteins (data not shown). Because of the relatively low yields of TAA90K obtained using this approach (30-50 μg/L media)

and significant TAA90K loss in the centrifugation devices, an alternate approach was explored to increase TAA90K yields.

5.2.2 Purification of TAA90K-His from HT-29 cells infected with recombinant vaccinia virus

In order to increase the expression of TAA90K in HT-29 cells and facilitate protein purification, we constructed a recombinant vaccinia virus expressing TAA90K-His containing a C-terminal poly-histidine tag suitable for purification by metal chelation affinity chromatography. The use of a recombinant vaccinia virus was desirable because it has the capability of infecting a wide range of mammalian cell lines and it results in the expression of recombinant protein in every infected cell with the proper post-translational modifications (Moss, 1996).

5.2.2.1 Generation of recombinant vaccinia virus

The first step in the preparation of recombinant vaccinia virus consisted of constructing a cDNA encoding TAA90K with an in-frame insertion of a C-terminal poly-histidine tag to create pCDTAA90-His. To examine the effect of the C-terminal histidine tag on TAA90K conformation, COS-1 cells were transfected with either pCD90, which contains the cDNA encoding wild-type TAA90K or pCDTAA90-His. Twenty-four hours later, transfected cells were radiolabeled overnight with [^{35}S]-methionine and the conditioned medium was immunoprecipitated using a panel of 12 TAA90K-specific monoclonal antibodies (Laferté *et al*., 2000). Both TAA90K-His and wild-type TAA90K were immunoprecipitated equally well with all of the antibodies (data not shown), suggesting that the conformation of TAA90K-His, relative to wild-type TAA90K, was not grossly altered by the presence of the histidine tag. Thus, we proceeded with the construction of a recombinant vaccinia virus encoding TAA90K-His. This was achieved by disrupting the thymidine kinase (TK) gene in wild-type vaccinia virus with the TAA90K-His coding sequence via homologous recombination. Rat-2 cells infected with individual recombinant vaccinia clones were tested for the expression of TAA90K-His by indirect immunofluorescence using TAA90K or His-tag specific monoclonal antibodies. Figure 5.4 shows an example of Rat-2 cells expressing

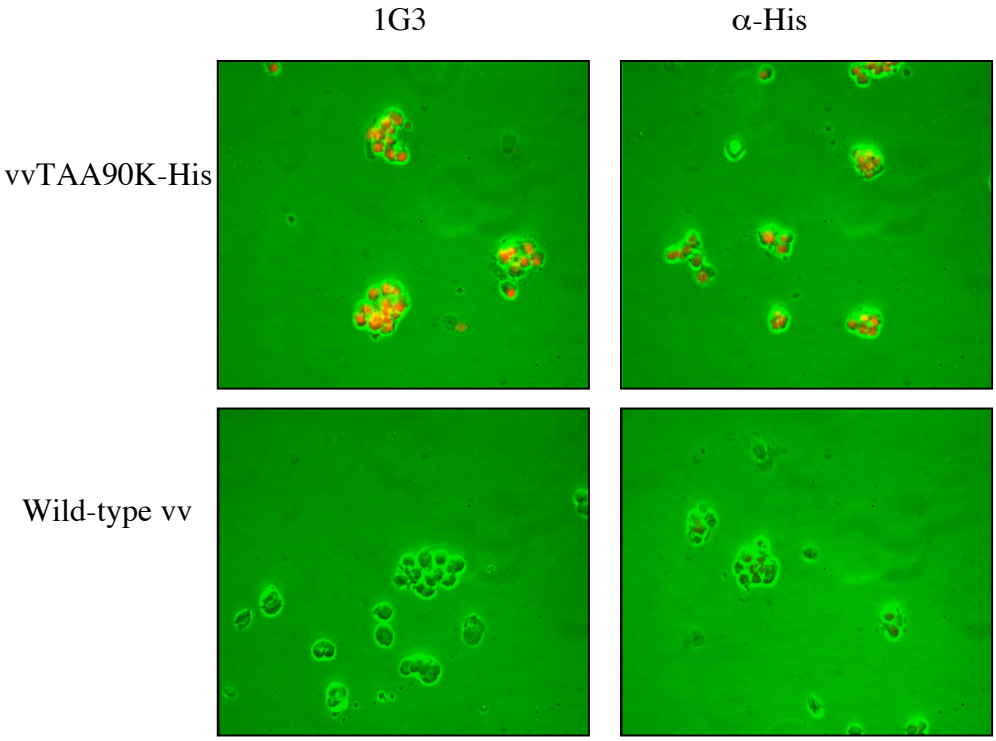


Figure 5.4: Indirect immunofluorescence of Rat-2 cells infected with wild-type vaccinia virus or recombinant vaccinia virus expressing TAA90K-His. Fixed and permeabilized Rat-2 cells infected with either wild-type (vv) or recombinant vaccinia virus (vvTAA90K-His) were incubated with a 1:250 dilution of TAA90K-specific monoclonal antibody 1G3 or mouse anti-His tag antibody. This was followed by incubation with secondary antibody FITC-goat anti-mouse IgG (1:50).

recombinant TAA90K-His following infection with one of the recombinant vaccinia clones isolated.

5.2.2.2 Optimization of purification strategies

TAA90K-His was purified initially from the conditioned medium of HT29 cells infected with recombinant vaccinia virus encoding TAA90K-His by metal chelation affinity chromatography employing His-bind resin. This method of purification proved to be inadequate for capturing TAA90K-His since the majority of the glycoprotein was found in the flow through fraction of the column. The lack of binding of TAA90K-His to the column could be due to the extensive glycosylation and oligomerization of TAA90K monomers that limit accessibility of the histidine tag to the His-bind resin. As a result, only a small amount of TAA90K-His (30-50 μg/L medium) was eluted from the column. In addition, the eluted fraction contained a number of additional proteins, thereby necessitating additional purification steps (Figure 5.5).

As a result, alternate chromatographic methods were evaluated for their ability to purify TAA90K-His to homogeneity. Chromatographic methods used successfully for the isolation of TAA90K-His consisted of lectin-affinity chromatography on wheat germ agglutinin (WGA)-agarose followed by cation- and anion-exchange chromatography. As expected, affinity chromatography on WGA-agarose was very effective at enriching for TAA90K-His. Although the HiTrap SP cation-exchange resin used as a second step did not bind TAA90K-His eluted from the previous WGA-agarose chromatography step, it did remove a number of contaminating proteins from the TAA90K-His enriched sample, including fibronectin. Finally, purified TAA90K-His was eluted from the HiTrap Q anion-exchange column in a single peak at approximately 0.6 M sodium chloride (NaCl) following elution with a gradient of 0.2 M to 1 M NaCl (Figure 5.6). Using this strategy, TAA90K-His was purified to near homogeneity (400-500 μg/L medium), as indicated by the single band migrating in SDS-PAGE with an apparent mobility of 105 kDa (Figure 5.7). Its identity as Mac-2-binding protein was confirmed by mass spectrometry. The minor species migrating at 82 kDa was shown by mass spectrometry to correspond to a proteolytic cleavage product of TAA90K-His. No other bands were detected in the gel.

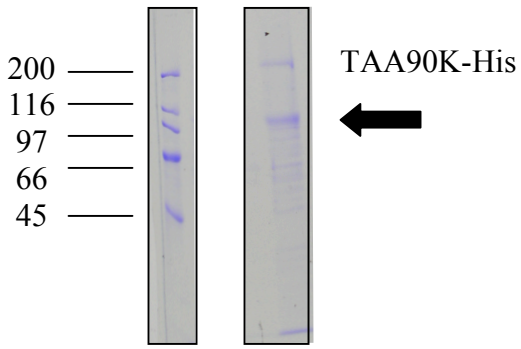


Figure 5.5: Purification of TAA90K-His from HT-29 conditioned medium employing metal chelation chromatography. SDS-PAGE analysis of TAA90K-His isolated from the conditioned medium from HT-29 cells infected with recombinant vaccinia virus encoding TAA90K-His employing metal chelation chromatography on His-bind resin. Molecular weight markers are indicated in kilodaltons at the left of the figure.

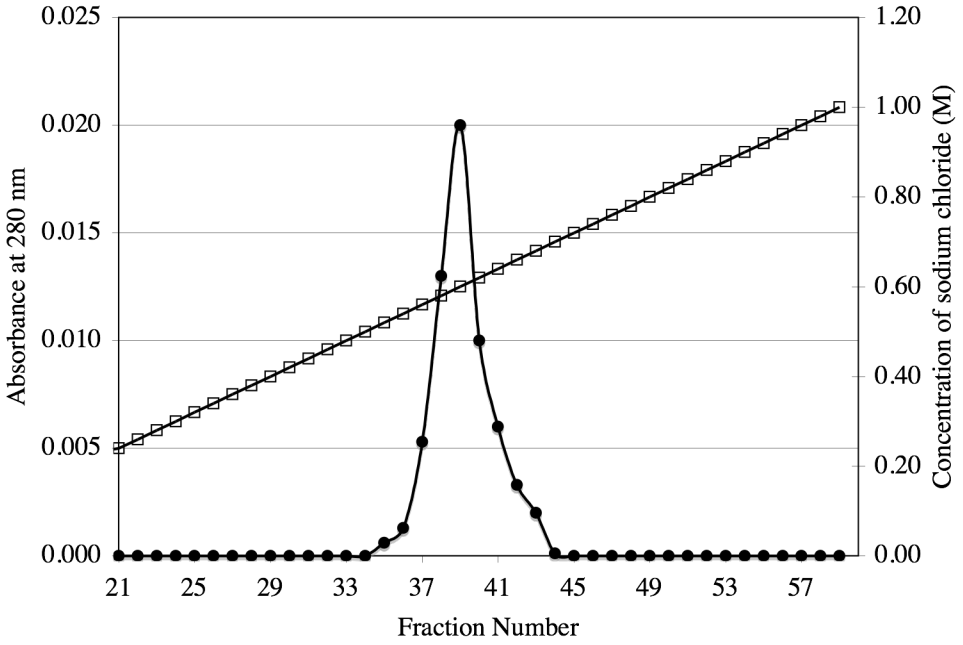


Figure 5.6: Elution profile of TAA90K from an anion-exchange column. Following lectin-affinity and cation-exchange chromatography, TAA90K or TAA90K-His were eluted from the anion-exchange column HiTrap Q HP with a gradient of 0.2 M to 1.0 M sodium chloride (open squares). The absorbance at 280 nm (closed circles) of the eluted fractions is shown.

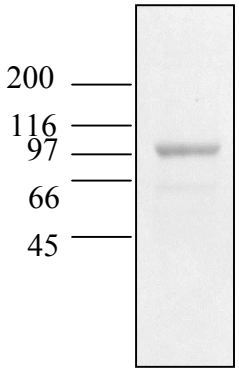


Figure 5.7: Analysis of purified TAA90K by SDS-PAGE. Coomassie-stained gel showing 1 μg of TAA90K-His purified from conditioned media of HT-29 cells infected with recombinant vaccinia virus expressing TAA90K-His. The size of the molecular weight markers in kilodaltons is shown at the left side of the figure.

Initial functional studies described below were carried out with TAA90K-His. However, since the presence of the poly-histidine tag did not aid in the purification of TAA90K, the majority of the functional studies described in this thesis were carried out with TAA90K purified from HT-29 cells infected with recombinant vaccinia virus expressing wild-type TAA90K. In side by side comparisons, TAA90K-His and TAA90K behaved identically in functional assays.

5.3 EXAMINATION OF TAA90K FUNCTION

5.3.1 TAA90K-PROTEIN INTERACTIONS

The immunohistochemical analysis of colon tumors revealed an elevated extracellular accumulation of TAA90K relative to normal mucosa. These findings, as well as the reported role of TAA90K as an adhesive protein mediating both cell-cell and cell-extracellular matrix interactions, suggested that TAA90K functions by interacting with extracellular proteins (Sasaki *et al*., 1998). We employed solid-phase binding assays to examine the interaction of TAA90K with extracellular matrix proteins and galectins.

5.3.1.1 Binding of TAA90K to ECM proteins

TAA90K purified from HT-29 cells bound poorly to collagens I and IV (Figure 5.8, panel A), however significant binding was observed to the extracellular matrix proteins laminin-1 (Figure 5.8, panel B) and fibronectin (Figure 5.8, panel B) as was shown previously for TAA90K isolated from EBNA-293 cells (Sasaki *et al*., 1998). Previously unidentified interactions between TAA90K and laminins 5 and 10 (Figure 5.8, panel B) were also detected.

5.3.1.2 Binding of TAA90K to galectins

TAA90K is a highly glycosylated protein capable of engaging in both protein-protein or protein-carbohydrate interactions (Sasaki *et al.,* 1998; Koths *et al.,* 1993; Müller *et al*., 1999). In addition to being modified by β1-6 linked oligosaccharides, TAA90K is also modified by poly-N-acetyllactosamine structures (repeating Galβ1-4GlcNAc units) (Laferté and Loh, 1992; Laferté *et al*., 2000). These oligosaccharides

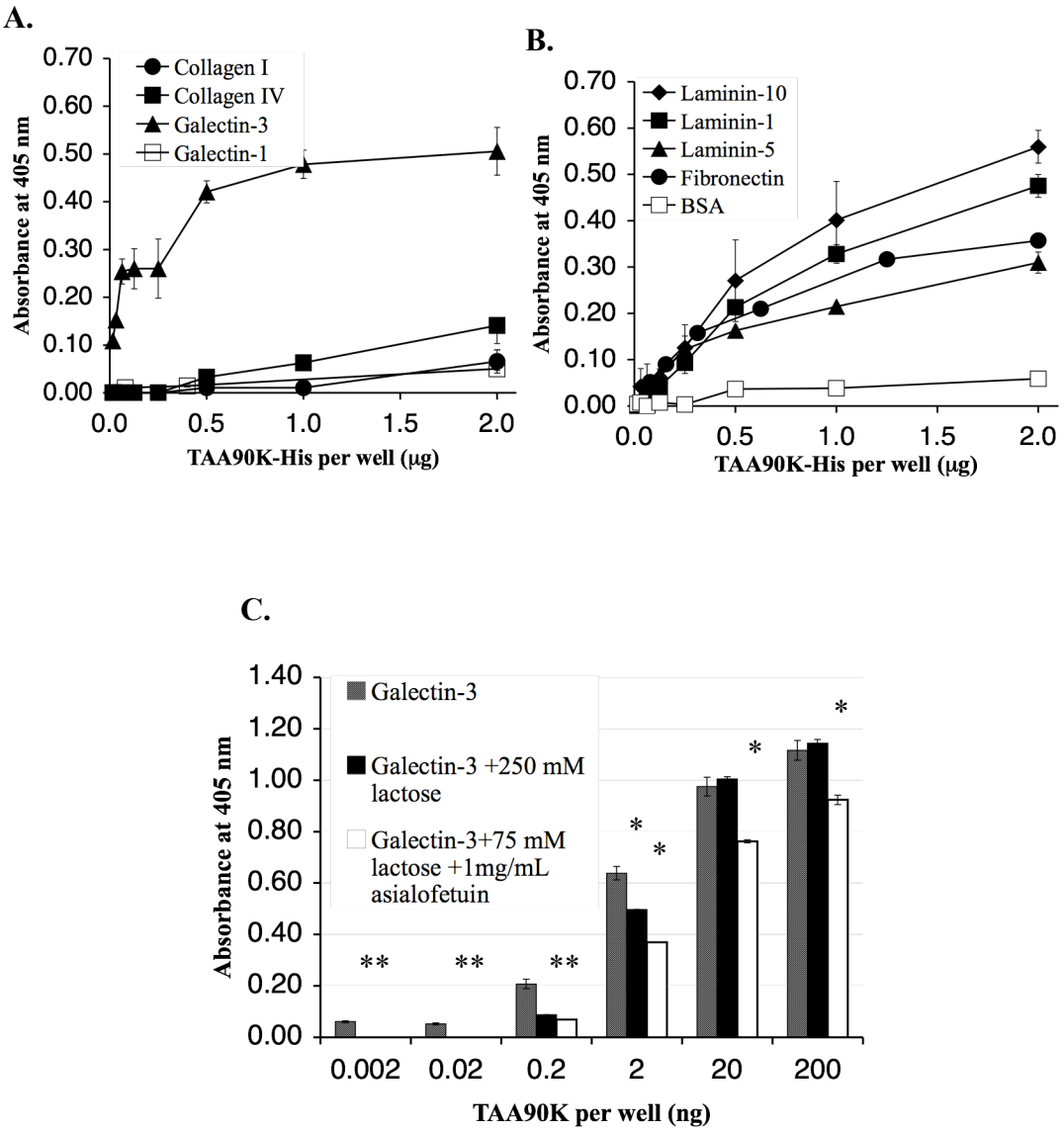


Figure 5.8: Binding of TAA90K-His to immobilized galectin-3 and extracellular matrix proteins and galectins. For experiments shown in **panels A** and **B**, wells were coated with the following proteins at 0.5 μg/ml except for fibronectin, which was used at 1 μg/ml. **Panel A**: collagen I (closed circle), collagen IV (closed square), galectin-1 (open square), galectin-3 (closed triangle); **Panel B**: laminin-1 (closed square), laminin-5 (closed triangle), laminin-10 (closed diamond), fibronectin (closed circle), BSA (open square). For the experiment shown in **panel C**, wells were coated overnight with galectin-3 alone (0.2 μg/ml; gray bar) or galectin-3 followed by 250 mM lactose (black bar) or 75 mM lactose containing 1 mg/ml asialofetuin (white bar). After washing and blocking, all wells (panels A-C) were incubated with increasing amounts of TAA90K-His (with or without lactose and asialofetuin), followed by MAb 1H9 and alkaline phosphatase-labeled goat anti-mouse Ig. Specific binding of TAA90K was detected spectrophotometrically at 405 nm. The mean and standard deviation of triplicate samples are shown. The asterisk indicates statistical significance with a *P*-value less than 0.05.

are potential binding sites for galectin-3, a dominant ligand for TAA90K (André *et al.,* 1999).

As expected, TAA90K purified from HT-29 cells bound well to galectin-3 (Figure 5.8, panel A). In contrast, TAA90K was shown to have a lower affinity for galectin-1. The reduced binding of TAA90K to galectin-1 could be due to cell-type-specific glycan structures found on TAA90K isolated from HT-29 cells. In collaboration with Dr. Hans-Joachim Gabius (Ludwig-Maximillians University), we examined the interaction of TAA90K with galectins -2, -4, -7 and -9, which revealed levels of binding of 0.6%, 27.4%, 63.0% and 32.7% relative to galectin-3.

In order to assess the contribution of complex-type oligosaccharides to TAA90K-galectin-3 binding, we examined TAA90K-galectin-3 binding in the presence of carbohydrates that compete for galectin-3 binding. To this end, either 250 mM lactose or 75 mM lactose and 1mg/mL of the glycoprotein asialofetuin, which contains di- and triantennary complex-type N-linked glycans containing N-acetyllactosamine (Galβ1-4GlcNAc) were added to the solid-phase binding assay. The data revealed that the interaction between TAA90K and galectin-3 was mediated in part by binding of galectin-3 to glycan structures on TAA90K, because the addition of either lactose alone or lactose in combination with asialofetuin, significantly reduced their interaction (Figure 5.8, panel C) ($P<0.05$).

An alternative approach to determine the effect of carbohydrates on TAA90K-galectin-3 interaction involved the use of a glycosylation inhibitor to generate an alternate glycoform of TAA90K. TAA90K was purified from HT29 cells treated with DMJ which blocks Golgi α-mannosidase I, the enzyme which prevents conversion of high-mannose type to complex-type N-linked oligosaccharides (Elbein *et al*., 1984). Evidence in support of altered carbohydrates on TAA90K from cells treated with DMJ (TAA90K-DMJ) included a smaller size on SDS-PAGE compared to wildtype TAA90K (88 kDa vs. 105 kDa) (Figure 5.9), as well as loss of binding to WGA-Sepharose and retention by cation-exchange resin, possibly facilitated by loss of the negatively charged sialic acid residues present on TAA90K (Laferté and Loh, 1992). In collaboration with Dr. Hans-Joachim Gabius, we analyzed the binding affinity between galectin-3 and TAA90K-DMJ. TAA90K-DMJ bound poorly to galectin-3 compared to

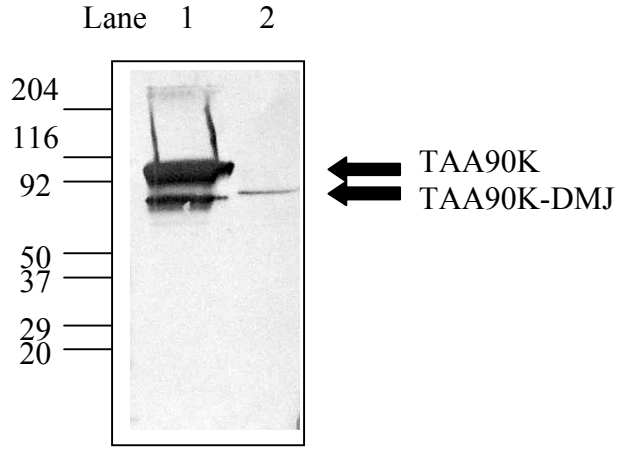


Figure 5.9: Western blot analysis of TAA90K isolated from HT29 cells treated with DMJ. TAA90K purified from HT29 cells untreated (lane 1) or treated (lane 2) with DMJ and infected with recombinant vaccinia virus expressing TAA90K was resolved on an SDS-PAGE gel. TAA90K was detected by Western blotting analysis using rabbit anti-TAA90K (1:5000), followed by alkaline-phosphatase conjugated goat anti-rabbit IgG second antibody (1:3000). The size of the molecular weight markers in kilodaltons is shown at the left side of the figure.

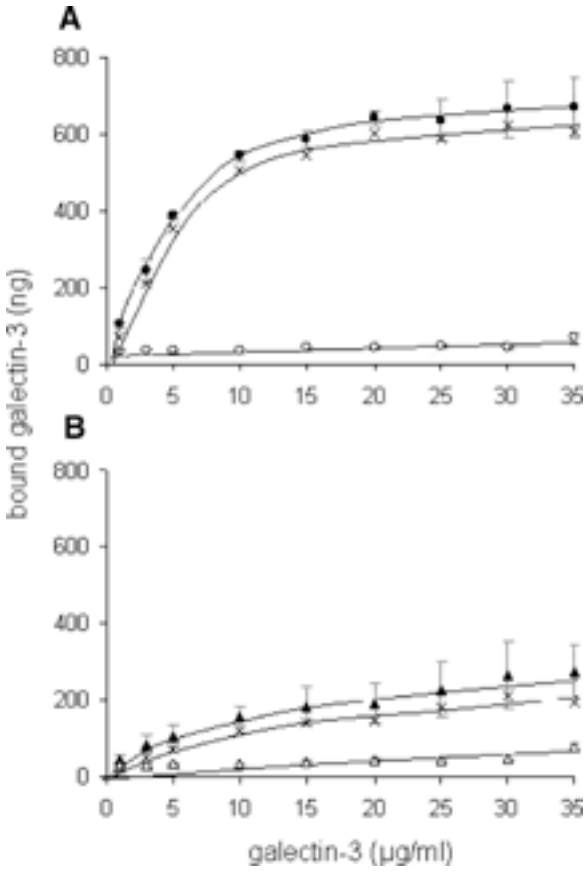


Figure 5.10: Effect of type of N-linked glycosylation on TAA90K binding to galectin-3. The level of carbohydrate-inhibitable binding (×) of biotinylated human galectin-3 to surface-immobilized TAA90K (**A**) and its variant from 1-deoxymannojirimycin-treated HT-29 cells (**B**) was determined using a solid-phase assay. Binding was detected spectrophotometrically at 490 nm. Total binding (closed circle, closed triangle) to the matrix using 100 ng glycoprotein per well for adsorption was reduced by the extent of carbohydrate-independent binding (open circle, open triangle) determined in the presence of a mixture of 75 mM lactose and 1 mg asialofetuin/ml as inhibitor (crosses).

wild-type TAA90K, as expected from the carbohydrate specificity of galectin-3 (Figure 5.10). These results indicated that the carbohydrates present on wild-type TAA90K play a role in mediating its interaction with galectin-3.

5.3.2 TAA90K-MATRIX METALLOPROTEINASE INTERACTIONS

The extracellular localization of TAA90K and its observed binding to extracellular matrix proteins suggested that TAA90K may play a role in restructuring of the ECM, which involves rebuilding and degradation of ECM components. In support of this hypothesis, Bair *et al*. (2006) demonstrated that addition of TAA90K to prostate cancer cells resulted in increased extracellular expression of promatrilysin (proMMP-7) in the culture medium. The enhanced expression of proMMP-7 by prostate cancer cells grown in the presence of TAA90K was shown to be mediated in part by IL-6, since cells grown in the presence of neutralizing antibodies to IL-6 exhibited reduced expression of proMMP-7 (Bair *et al.,* 2006). In light of these findings, we undertook to examine the effect of TAA90K on expression of proMMP-7 in colon cancer cells, as well as the interaction of TAA90K with MMPs.

5.3.2.1 Effect of TAA90K on expression of proMMP-7 in HT-29 cells

In order to determine if TAA90K has any effect on the expression of active and proMMP-7 in the conditioned medium of HT-29 cells, increasing amounts of TAA90K were added to cells grown in serum-free medium. The medium was concentrated 5-fold and tested by ELISA with a monoclonal antibody against pro-MMP-7. The results revealed that addition of 1 μg TAA90K to HT-29 cells enhanced expression of proMMP-7 in the conditioned medium 24 h ($P<0.005$) and 48 h ($P<0.0005$) post-treatment, compared to untreated cells. Furthermore, the addition of 5 μg TAA90K to HT-29 cells significantly increased the expression of proMMP-7 at 24 h ($P<0.05$), 48 h ($P<0.005$) and 72 h ($P<0.005$) post-treatment (Figure 5.11). There was no change in the extracellular expression of active MMP-7 from HT-29 cells grown in the presence or absence of TAA90K (data not shown).

In addition to examining the effect of TAA90K on proMMP-7 extracellular expression in HT-29 cells, we also examined the expression of pro- and active forms of

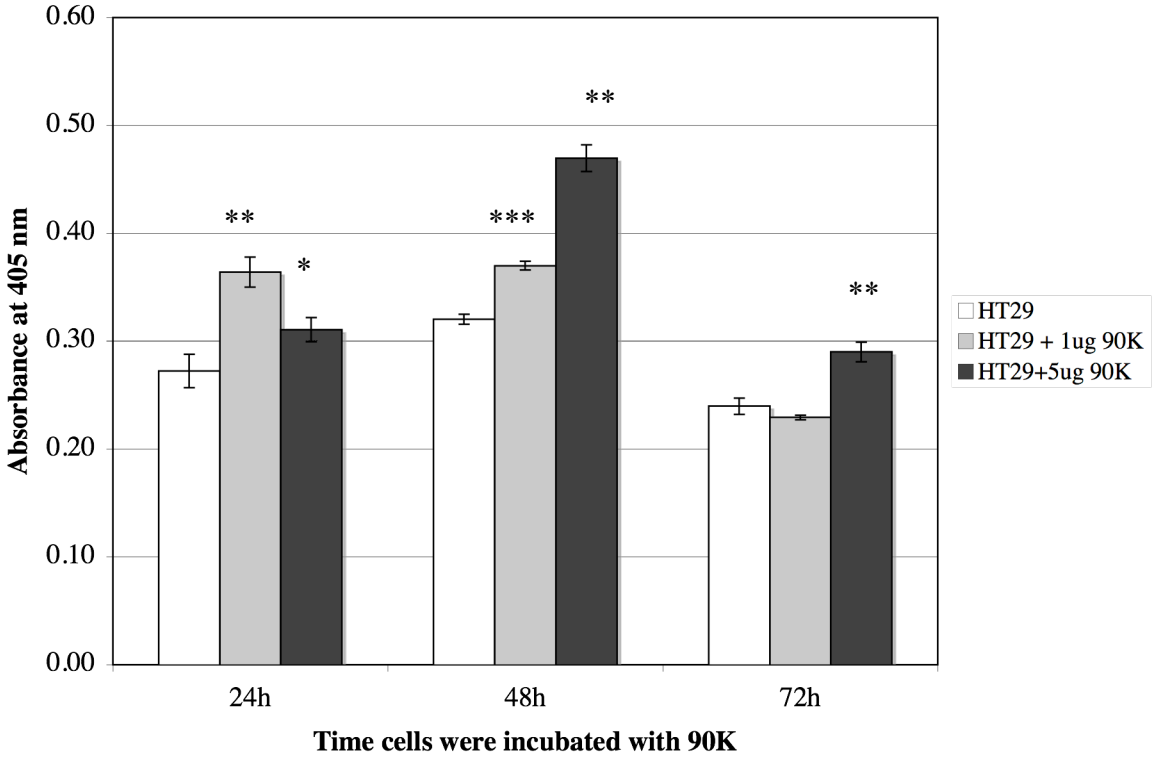


Figure 5.11: ELISA of proMMP-7 expression from HT-29 cells grown in the presence or absence of TAA90K. HT-29 cells were incubated in serum-free medium containing 0 μg, 1 μg or 5 μg TAA90K for 24h, 48h or 72h. The medium was concentrated 5-fold and 100 μL was added in triplicate to the wells of a 96 well plate. The amount of proMMP-7 was detected by incubation with mouse anti-proMMP-7 antibody or NMS (1:1000) as a control, followed by alkaline-phosphatase-conjugated goat anti-mouse IgG second antibody (1:3000). Binding was detected spectrophotometrically at 405 nm. The data shown represents the specific binding of anti-proMMP-7 antibody. The mean and standard deviation of triplicate experiments coated in triplicate (n=9) are shown. The asterisk (s) indicates statistical significance with a *P*-value less than 0.05 (*), 0.005 (**) or 0.0005 (***).

MMP-7 in the serum-free conditioned medium of a panel of human colon cancer cell lines, including Caco-2, HT-29, KM20C, LoVo, LS-123, SW403 and SW620. To this end, the medium was concentrated 5-fold and subjected to an ELISA to measure the levels of pro- and active MMP-7 expression (Figure 5.12). The ELISA detected small amounts of active MMP-7 expression in all cell lines examined and variable expression of proMMP-7, with maximal expression observed in KM20C and HT-29 cell lines.

5.3.2.2 Binding of TAA90K to MMPs and regulators of MMP activity or expression

In an attempt to elucidate the mechanism involved in the enhanced expression of proMMP-7 in the conditioned medium of HT-29 cells treated with TAA90K, we performed solid phase binding assays to examine whether TAA90K binds directly to either the proenzyme or active forms of MMP-7. TAA90K bound well to both the pro- and active forms of MMP-7, but poorly to proMMP-1 (Figure 5.13). This suggested that extracellular binding of TAA90K to pro- or active forms of MMP-7 may stabilize MMP-7 and/or protect it against proteolytic degradation. This could account for the increased level of proMMP-7 detected in the conditioned medium of TAA90K-treated HT-29 cells. On the other hand, no detectable binding of TAA90K to the active (data not shown) or proenzyme forms of MMP-2 and -9, two MMPs shown to play a significant role in colon cancer progression (reviewed in Zucker and Vacirca, 2004) was observed.

Alternatively, TAA90K could block the interaction between MMP-7 and its inhibitors, including tissue inhibitors of matrix metalloproteinases (TIMPs) and α_2-macroglobulin (reviewed in Zucker and Vacirca, 2004; Sottrup-Jensen and Birkedal-Hansen, 1989). Thus, we examined whether TAA90K binds to inhibitors of MMPs as a possible mechanism for increased expression. Our results indicated that TAA90K does not bind to either TIMP -1, -2 or $\alpha_2$macroglobulin (data not shown), suggesting that TAA90K does not affect MMP-inhibitor interactions directly. However, it is possible that TAA90K and TIMPS could compete for binding to the same site on the enzyme.

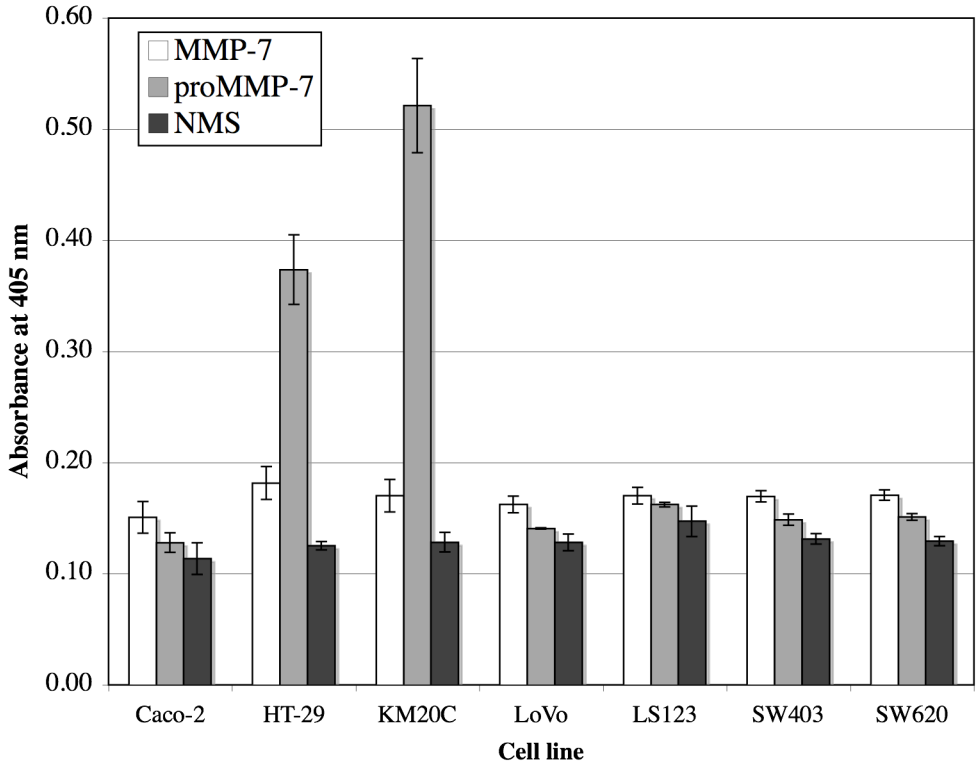


Figure 5.12: ELISA analysis of pro- and active MMP-7 secretion by human colon cancer cell lines. Conditioned serum-free medium from human colon cancer cells was concentrated 5-fold and coated (100 μL) in triplicate in a 96 well plate. Wells were washed, blocked with TBS containing 4% BSA, washed and incubated with monoclonal antibodies against proMMP-7, active MMP-7 or normal mouse serum (1:1000) for 2 h at room temperature. After washing, wells were incubated with alkaline phosphatase-conjugated goat anti-mouse Ig (1:3000) for 1h followed by washing and addition of substrate. Plates were read at 405 nm. The mean and standard deviation of triplicate samples are shown.

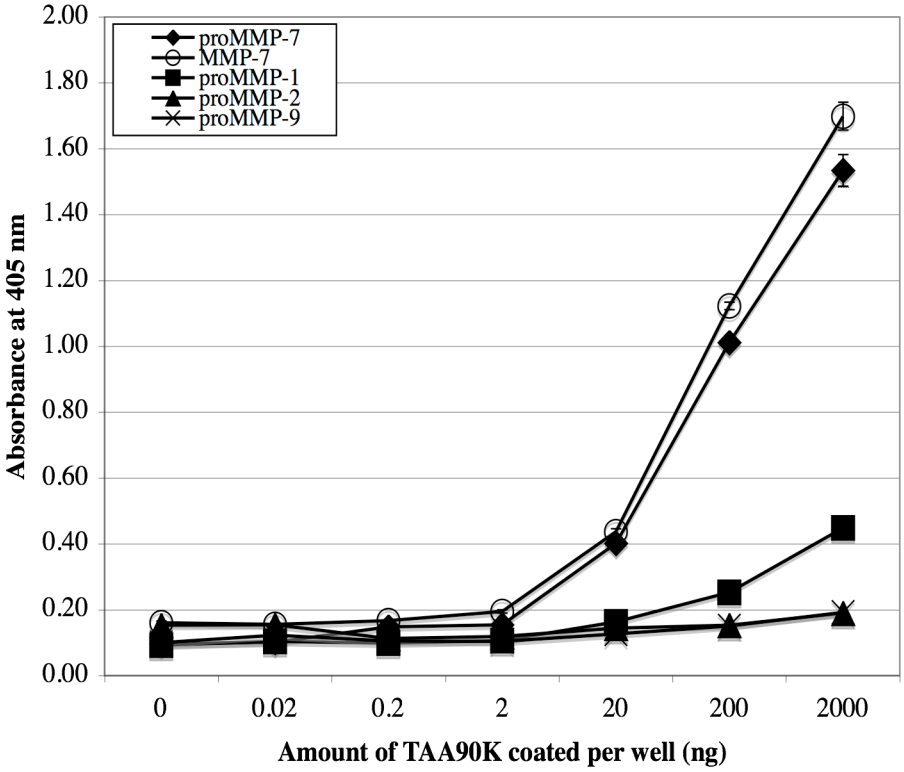


Figure 5.13: Solid-phase binding assay to examine binding of TAA90K to MMPs. Substrates including proMMP-1 (square), proMMP-2 (triangle), proMMP-7 (diamond), MMP-7 (circle) and proMMP-9 (X) were coated (0.5 μg) overnight in triplicate in the wells of a 96 well plate. After washing, blocking in BSA, and washing again, different concentrations of TAA90K were added to the wells and incubated overnight. Following washing and incubation with MAb 1H9 (1:1000), a goat anti-mouse alkaline phosphatase-conjugated second antibody (1:3000) was added. After washing and developing, the assays were read at 405 nm. Error bars represent standard deviation.

Lastly, we examined whether TAA90K binds to proteins implicated in signaling events leading to enhanced proMMP-7 expression. Previous studies have shown that the secretion of proMMP-7 by prostate cancer cells requires the binding of IL-1β to its cell surface receptor. This results in activation of the transcription factor NFκB and subsequent expression of IL-6. Enhanced secretion of IL-6 in turn leads to binding to its cell surface receptor (IL-6R), initiating signaling events that result in elevated expression of proMMP-7 (Maliner-Stratton *et al.,* 2001). In light of these data, we examined whether TAA90K can interact directly with IL-6 or IL-1β as a means to modulate receptor-mediated signaling events. Using solid-phase binding assays, we determined that intact TAA90K interacts with both IL-6 and IL-1β in a concentration-dependent manner (Figure 5.14). Thus, the TAA90K-enhanced extracellular expression of proMMP-7 from HT-29 cells may be mediated in part by its interactions with both IL-6 and IL-1β.

5.3.2.3 Proteolytic cleavage of TAA90K by MMP-7

Matrix metalloproteinases cleave a wide range of substrates, which range from ECM proteins such as laminins, fibronectin and collagens to non-ECM proteins such as growth factors like TGF-β (McCawley and Matrisian, 2001). Recently, Canals *et al.* (2006) identified TAA90K as a potential target for proteolysis by ADAMTS1, a metalloproteinase similar to, but not in the same family as MMPs. In light of these data, we examined whether TAA90K is a substrate for MMP-7. When MMP-7 and TAA90K (1:50, w/w, respectively) were incubated for 24 h at 37 ^{o}C, TAA90K was cleaved to fragments of 79 kDa and 22 kDa (Figure 5.15, panel A). Western blotting analysis of these digests using rabbit anti-TAA90K confirmed that both fragments originated from TAA90K (Figure 5.15, panel B). The minor bands present in the Western blot are likely the result of alternate cleavage sites.

5.3.2.3.1 Purification of proteolytic fragments

To test whether cleavage of TAA90K by MMPs alters its function, we purified cleaved TAA90K for use in functional assays and identification of the amino acid cleavage site (s). To this end, TAA90K was cleaved with MMP-7 and purified by

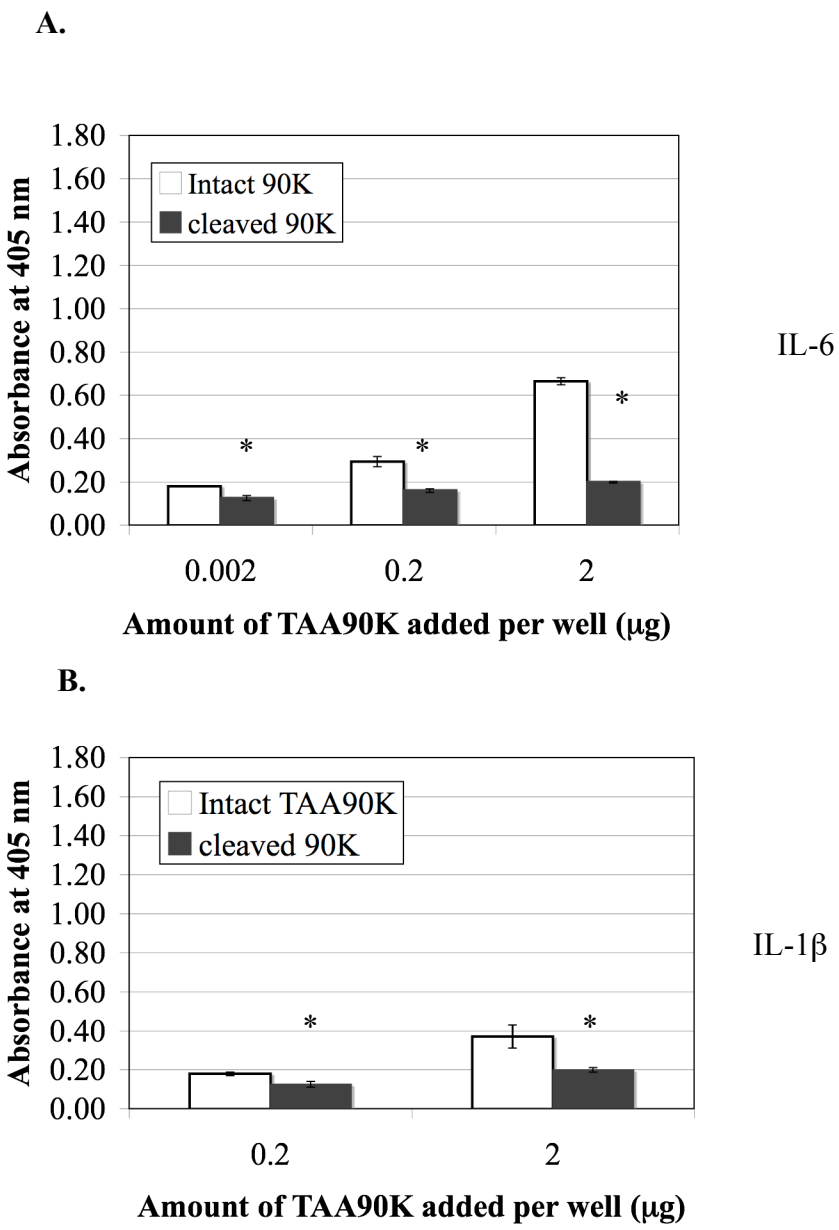


Figure 5.14: Solid-phase binding assay to examine binding of intact TAA90K and MMP-7-cleaved TAA90K to IL-6 and IL-1β. IL-6 (panel A) or IL-1β (panel B) were coated (0.5 μg) overnight in triplicate in the wells of a 96 well plate. Following washing and blocking, either intact TAA90K or MMP-7-cleaved TAA90K were incubated overnight. Following washing, incubation with MAb 1H9 (1:1000) and alkaline-phosphatase-labeled goat anti-mouse Ig (1:3000), specific binding of TAA90K was detected spectrophotometrically at 405 nm. Error bars represent standard deviation and the asterisks (*) indicate $P<0.05$.

anion-exchange chromatography on HiTrap Q HP resin to remove the enzyme from the cleaved TAA90K preparation (Figure 5.15, panel A). Using this approach, the enzyme eluted in the run-through fraction, whereas TAA90K eluted in 1M NaCl. The cleaved TAA90K sample was desalted on a NAP-5 column and used for functional studies. Western blotting analysis of cleaved TAA90K revealed that the 79 kDa and 22 kDa fragments co-eluted from the anion-exchange column, suggesting that the cleaved fragments remain associated within the oligomeric complex.

To identify the proteolytic cleavage site on TAA90K, MMP-7 treated TAA90K was separated by SDS-PAGE, transferred to a PVDF membrane, stained with Ponceau Red and the excised bands corresponding to the 79 kDa and 22 kDa fragments were submitted for protein sequencing analysis (Harvard Microchem Facility, Boston, MA). N-terminal sequencing analysis of the 79 kDa fragment by Edman degradation revealed a 7- amino acid peptide sequence, VNDGDMRA, identical to the N-terminal sequence of intact TAA90K (Koths *et al.,* 1993). Similar analysis of the 22 kDa fragment revealed an N-terminal peptide sequence of YRYYPYQSFQ, consistent with an internal cleavage of the TAA90K sequence at $Asp^{452}Tyr^{453}$. There was also evidence of minor cleavage sites at $Pro^{450}Ser^{451}$ and $Gln^{462}Thr^{463}$. C-terminal sequencing analysis of the 79 kDa fragment was also carried out to determine whether the 79 kDa and 22 kDa fragments generated by MMP-7 resulted from a single cleavage event. Mass spectrometry analysis of trypsin and endopeptidase LysC digests of the 79 kDa fragment identified Asp^{452} as the C-terminal residue, consistent with cleavage between Asp^{452} and Tyr^{453} (Figure 5.16).

5.3.2.4 Cleavage of TAA90K by other MMPs

In addition to examining the effect of MMP-7 on TAA90K cleavage, we also examined whether TAA90K is a substrate for MMP-2 and -9, two other MMPs that have been implicated in colon cancer progression (reviewed in Zucker and Vacirca, 2004). When MMP-2, MMP-7 or MMP-9 were each incubated with TAA90K in a ratio of 1:3 (w/w), respectively, for 1 h at 37 ^{o}C in the presence of zinc ions, this resulted in cleavage of

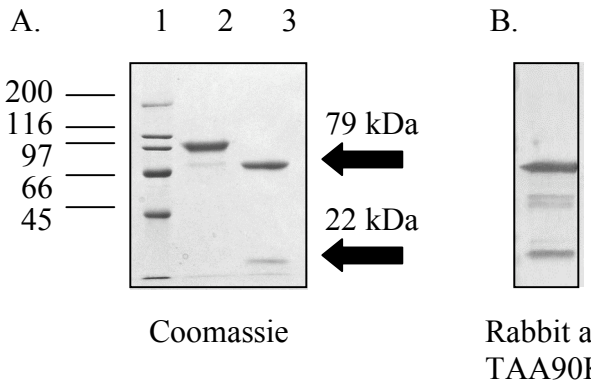


Figure 5.15: SDS-PAGE analysis of TAA90K cleaved by MMP-7. Panel A. TAA90K was incubated with (lane 3) or without (lane 2) MMP-7 at a substrate to enzyme ratio of 50:1 (w/w), respectively, for 24 h at 37^0C. Cleaved TAA90K was purified by anion-exchange chromatography. Four micrograms each of full length and cleaved TAA90K were resolved on a 10% SDS-PAGE gel and stained with Coomassie blue. **Panel B.** MMP-7-treated TAA90K was resolved on a 10 % SDS-PAGE gel, transferred to nitrocellulose and probed with rabbit anti-TAA90K (1:5000). The size of the molecular weight markers are indicated in kilodaltons at the left of the figure.

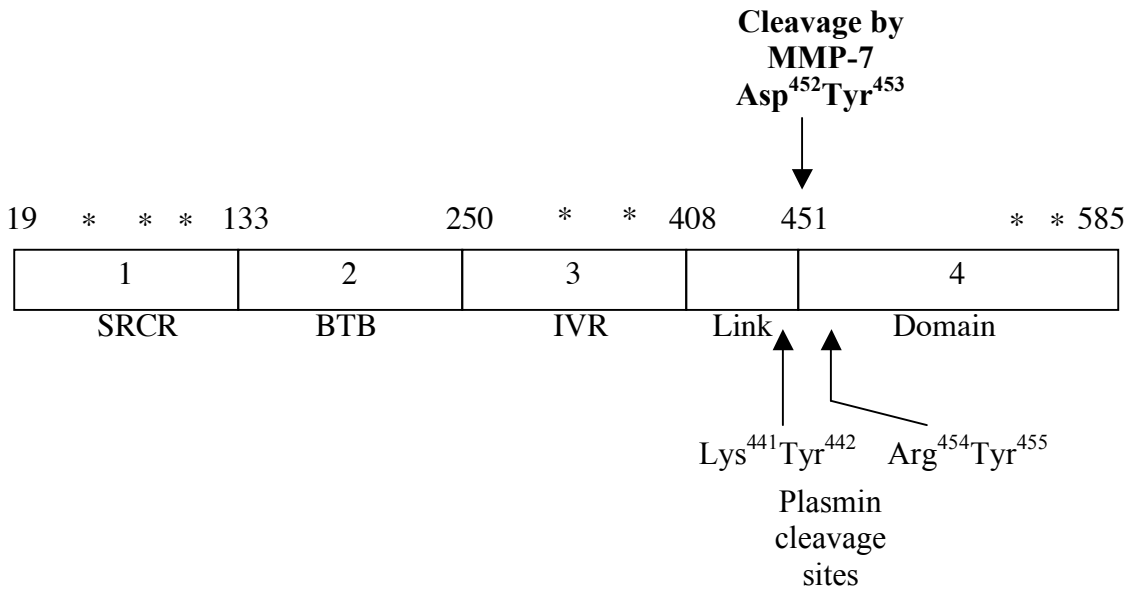


Figure 5.16: Schematic diagram of the putative domains of TAA90K indicating the major MMP-7 cleavage site. Putative domains are represented by boxes flanked by their respective amino acid residues. Potential asparagine-linked glycosylation sites are indicated by asterisks (*). Cleavage sites for plasmin and MMP-7 are indicated with arrows.

TAA90K into two major fragments of 79 kDa and 22 kDa (Figure 5.17). As expected for matrix metalloproteinases, the enzymatic cleavage was inhibited by 50 mM EDTA since it chelates the metal ions required for enzyme activity (reviewed in Zucker and Vacirca, 2004). Similarly, boiling of the enzyme at 100^{o}C for 4 minutes inactivated the enzyme and inhibited proteolytic cleavage.

5.3.2.5 Binding of MMP-7-cleaved TAA90K to extracellular proteins

Previous work including our own has shown that TAA90K binds to a number of proteins including fibronectin, laminin-1, laminin-5, laminin-10, galectin-3, IL-6, proMMP-7 and active MMP-7 (Sasaki *et al*., 1998; Ulmer *et al*., 2006). In addition, TAA90K is a substrate for MMP-7, yielding fragments of 79 kDa and 22 kDa. In order to elucidate the biological effect of TAA90K cleavage by MMP-7, we performed solid-phase binding assays to compare the binding of cleaved and intact TAA90K to extracellular proteins. Cleaved TAA90K showed similar binding compared to full length TAA90K for pro- and active MMP-7, laminin-1, collagen I, collagen IV and galectin-3 (data not shown). In contrast, binding of cleaved TAA90K to fibronectin was reduced significantly compared to intact TAA90K (Figure 5.18, panel A). These results suggested that the TAA90K domain responsible for interacting with fibronectin has been altered directly by cleavage with MMP-7 or indirectly through a conformational change resulting from the proteolytic cleavage. Similarly, binding of cleaved TAA90K to laminin-10 was reduced significantly compared to intact TAA90K (Figure 5.18, panel B). Lastly, MMP-7 cleavage of TAA90K resulted in significantly reduced binding to IL-6 and IL-1β compared to intact TAA90K (Figure 5.14). Interestingly, incubation of HT-29 cells with 5 μg TAA90K that had been cleaved by MMP-7 for 48 h revealed elevated proMMP-7 secretion compared to control cells, however, the results were significantly less than when 5 μg of intact TAA90K was added to cells (P<0.003) (Figure 5.19). This suggested that TAA90K may modulate the expression of proMMP-7 through its interaction with cytokines.

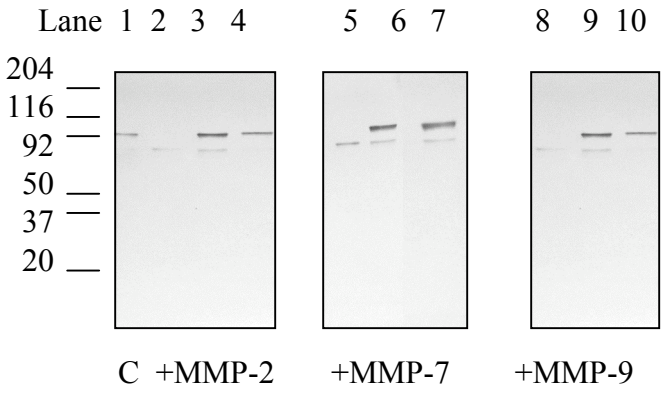
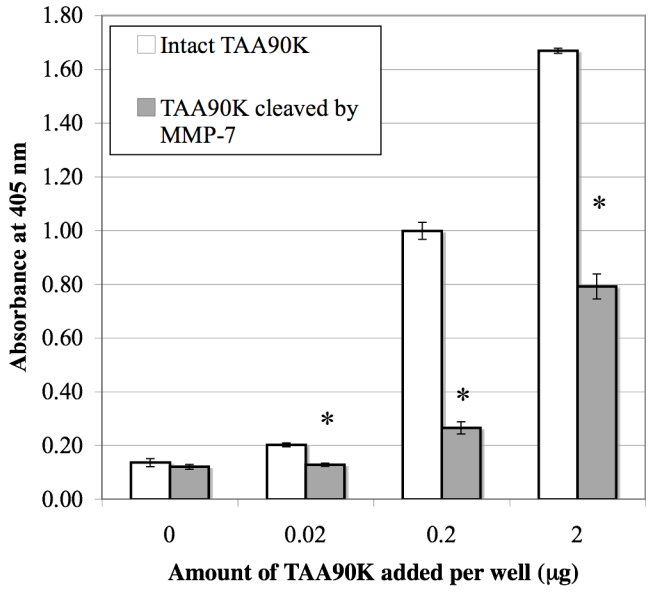


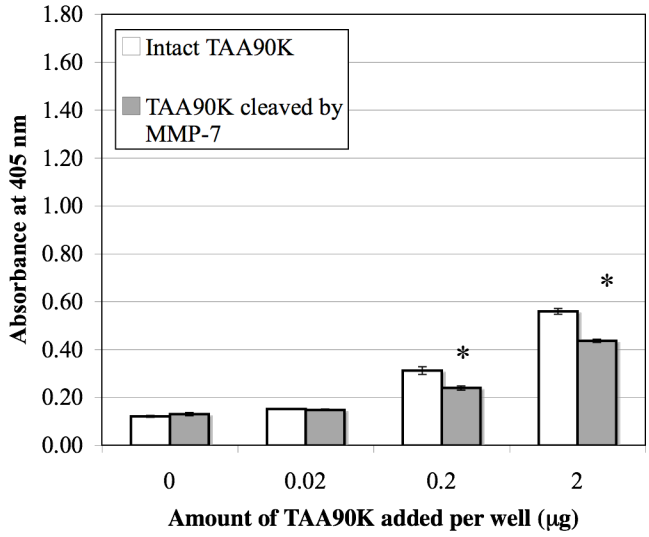
Figure 5.17: Western blotting analysis of TAA90K cleavage by MMP-2, -7 and -9. Active MMP-2 (lanes 2-4) or -7 (lanes 5-7) or -9 (lanes 8-10) were incubated with TAA90K at a ratio of 1:3 (w/w) at 37ºC for 1h. Prior to incubation, the enzymes used in lanes 4, 7 and 10 were boiled at 100ºC for 4 minutes. Digests in lanes 3, 6 and 9 were incubated in an inhibitory buffer containing 50 mM EDTA. Lane 1 contained untreated TAA90K (C). Digests were resolved on a 10 % gel and transferred to nitrocellulose. Western blotting analysis was performed with rabbit anti-TAA90K polyclonal antibody (1:5000). The size of the molecular weight markers in kilodaltons is shown at the left of the figure.

A.



Fibronectin

B.



Laminin-10

Figure 5.18: Solid-phase binding assay to examine the effect of TAA90K cleavage by MMP-7 on binding to extracellular matrix proteins. Fibronectin (**panel A**) or laminin-10 (**panel B**) were coated (0.5 μg/well) onto the wells of a 96-well plate overnight. Following washing and blocking, increasing amounts of either intact TAA90K or MMP-7-cleaved TAA90K were incubated, followed by MAb 1H9 and alkaline phosphatase-labeled goat anti-mouse Ig. Specific binding of TAA90K was detected spectrophotometrically at 405 nm. The mean and standard deviation of triplicate samples are shown. The asterisks indicate statistical significance with * representing a *P*-value<0.005.

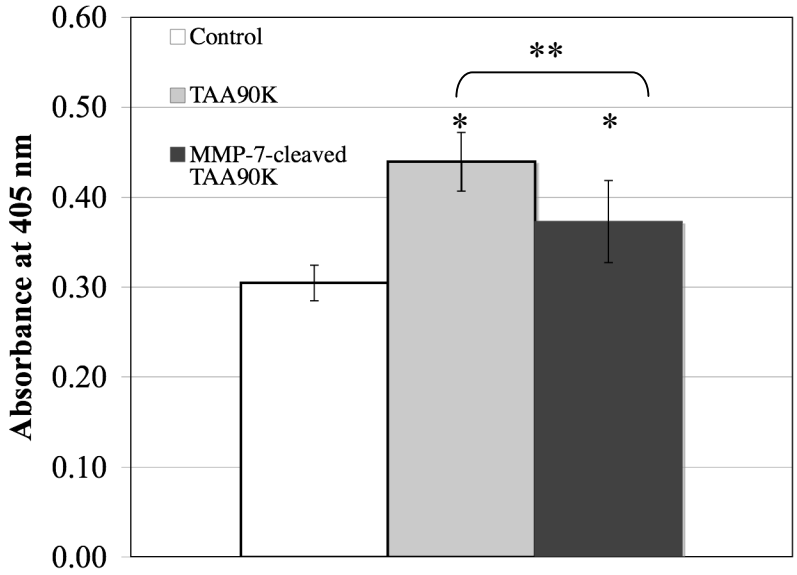


Figure 5.19: ELISA of proMMP-7 expression in conditioned medium from HT-29 cells grown in the presence or absence of intact TAA90K or MMP-7–cleaved TAA90K. HT-29 cells were incubated in serum-free media containing 0 μg (control) or 5 μg of intact or MMP-7-cleaved TAA90K for 48 h. Medium was concentrated 5-fold and 100 μL was added in triplicate to the wells of a 96 well plate. The amount of proMMP-7 was detected by incubation with mouse anti-proMMP-7 antibody or NMS (1:1000) as a control, followed by alkaline-phosphatase-conjugated goat anti-mouse IgG second antibody (1:3000). Binding was measured spectrophotometrically at 405 nm. The data shown represents the specific binding of anti-proMMP-7 antibody. The mean and standard deviation of two experiments coated in triplicate are shown (n=6). The single asterisk (*) indicates a statistical significance for the effect of intact and MMP-7-cleaved TAA90K compared to control samples with $P<0.001$. The double asterisk (**) indicates a statistical significance for the reduced extracellular expression of proMMP-7 following treatment of HT-29 cells with MMP-7-cleaved TAA90K ($P<0.003$).

5.3.3 EFFECT OF TAA90K ON TUMOR CELL PROPERTIES

5.3.3.1 Effect of TAA90K on cellular adhesion

Although the function of TAA90K in normal and neoplastic tissues is unclear, previous studies have implicated TAA90K as a multi-adhesive protein within the extracellular matrix, mediating binding to both ECM and cell surface receptors (Sasaki *et al.,* 1998). For this reason, the adhesiveness of human colon cancer cell lines to TAA90K isolated from HT-29 cells was examined using cellular adhesion assays. In contrast to previous findings where TAA90K purified from EBNA-293 cells was shown to mediate the adhesion of HBL-100 breast epithelial cells (Sasaki *et al*., 1998; Hellstern *et al*., 2002), TAA90K isolated from HT-29 cells did not mediate the adhesion of HT29 cells and six other human colon carcinoma cell lines (Caco-2, KM20C, LoVo, LS123, SW403 and SW620). Similarly, the normal rat intestinal cell line IEC-6, Rat-2 fibroblasts and HBL100 breast epithelial cells were unable to adhere to TAA90K (data not shown). The lack of binding of HT-29 cells was specific to TAA90K since these cells adhered to fibronectin, collagen I and galectin-3 (Figure 5.20). The lack of binding of TAA90K to HT-29 cells was confirmed by indirect immunofluorescence experiments which showed no staining of intact cells with TAA90K-specific monoclonal antibody 1H9 (Laferté and Loh, 1992) or following incubation of cells with purified TAA90K and MAb 1H9 (data not shown). It is possible that the oligosaccharides present on TAA90K synthesized by HT-29 inhibit TAA90K-mediated cell adhesion, in contrast to TAA90K isolated from other cell types (Sasaki *et al*., 1998).

Interestingly, initial cell adhesion experiments carried out with TAA90K purified from uninfected HT-29 cells (no recombinant vaccinia virus) did mediate HT-29 cell adhesion. However, it was later determined that the cell adhesion resulted from fibronectin contamination. When fibronectin was later removed from the TAA90K preparations by cation-exchange chromatography, a step not used by other investigators, we no longer observed cell adhesion to our TAA90K preparations. In light of these findings, it is possible that TAA90K preparations isolated from other cell types (Sasaki *et al*., 1998; Hellstern *et al.,* 2001) may have been contaminated with extracellular proteins, including fibronectin.

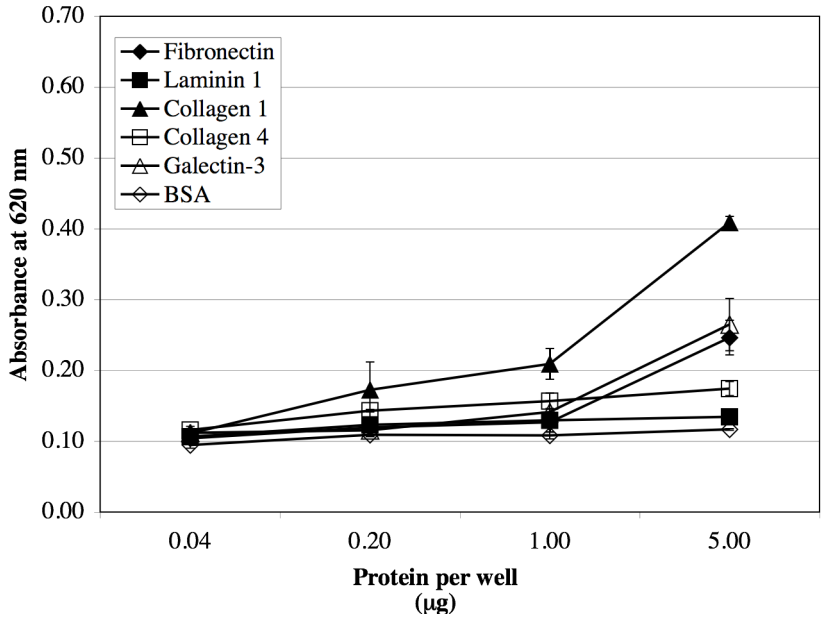


Figure 5.20: Adhesion of HT29 to various proteins. Wells were coated with extracellular matrix proteins, galectin-3 or BSA. After incubation with HT29 cells for 4 h, non-adherent cells were removed by washing. Adherent cells were fixed with formaldehyde and stained with toluidine blue. Adhesion of HT29 cells was detected spectrophotometrically at 620 nm. The mean and standard deviation of triplicate samples are shown.

5.3.3.1.1 Effect of altered cell surface and TAA90K glycosylation on cellular adhesion

The lack of cell adhesion to TAA90K may result from cell-type-specific glycosylation of TAA90K and/or its cellular receptor(s) resulting in an anti-adhesive rather than adhesive interaction. In order to assess the contribution of complex carbohydrates to TAA90K function, it was necessary to generate different glycoforms of TAA90K. Previous work from this laboratory showed that the molecular mass of TAA90K immunoprecipitated from the conditioned medium of different cell lines ranges from 90 kDa to 105 kDa (Laferté and Loh, 1992). These differences in molecular mass could be attributed to cell-specific glycosylation differences (Laferté and Loh, 1992). This meant that expression of TAA90K in different cell lines could yield different glycoforms of TAA90K. To this end, recombinant vaccinia virus containing the cDNA for TAA90K was used to infect rat intestinal IEC-6 cells in addition to the human colon cancer cell lines SW620 and SW480. The amount of TAA90K produced by these cells following infection with recombinant vaccinia virus was significantly less than that produced by similar treatment of HT-29 cells. As a result, it was not possible to purify sufficient quantities of recombinant TAA90K from these cell lines for functional studies.

An alternative approach to generating different glycoforms of TAA90K involved treating of HT-29 cells with the glycosylation inhibitors swainsonine (Sw) or 1-deoxymannonirimycin (DMJ) during infection with recombinant vaccinia virus. DMJ prevents the conversion of high mannose to complex-type N-linked oligosaccharides (Elbein *et al*., 1984). Swainsonine inhibits Golgi α-mannosidase II, which prevents the synthesis of β1-6 branched complex-type N-linked oligosaccharides (Elbein *et al*., 1981). This in turn reduces the level of poly-N-acetyllactosamine [$(Gal\beta1\text{-}4GlcNAc)_n$] structures (van den Eijnden *et al*., 1988). A TAA90K glycoform was purified from HT-29 cells treated with DMJ. Similar to fully glycosylated TAA90K, TAA90K-DMJ treated HT-29 cells also failed to mediate the adhesion of HT-29 cells or HT-29 cells treated with DMJ (data not shown). TAA90K isolated from vaccinia-infected HT-29 cells treated with swainsonine (TAA90K-Sw) contained a mixture of glycoforms, including fully glycosylated as well as truncated glycoforms. Similarly to TAA90K-

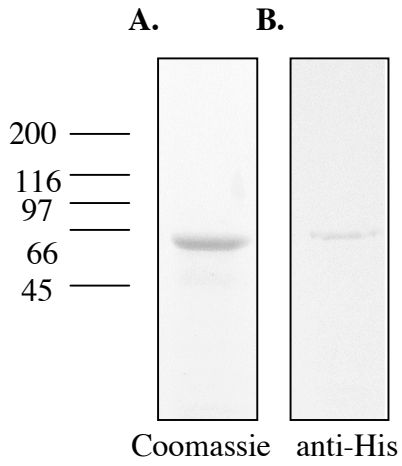


Figure 5.21: SDS-PAGE analysis of His-TAA90K isolated from recombinant bacteria. Coomassie-stained gel (**panel A**) and anti-His Western blot (**panel B**) of 1 μg of His-TAA90K purified from recombinant BL21 bacteria expressing His-TAA90K. The size of the molecular weight markers in kilodaltons is shown at the left side of the figure.

DMJ, TAA90K-Sw failed to mediate adhesion of HT-29 cells or HT-29 cells pre-treated with swainsonine (data not shown).

In an attempt to examine the overall contribution of complex carbohydrates to TAA90K function in biological assays, TAA90K devoid of glycan chains was purified from recombinant bacteria expressing His-TAA90K. The 60 kDa band shown in Figure 5.21 corresponds to the unglycosylated form of recombinant TAA90K containing an N-terminal histidine tag (His-TAA90K). As shown in Figure 5.22, the unglycosylated form of TAA90K also failed to mediate the adhesion of HT29 cells. However, treatment of HT29 cells with DMJ to alter cell-surface glycosylation resulted in enhanced cell binding to bacterially produced His-TAA90K (Figure 5.22). Thus, the adhesive interactions between TAA90K and its putative cellular receptor(s) may be modulated by substratum- and cell-type-dependent N-linked glycosylation.

5.3.3.2 Effect of TAA90K on cell adhesion to galectin-3

Although our data demonstrated that TAA90K does not mediate cell adhesion directly, we investigated whether TAA90K can modulate cell adhesion to other substrates. Since TAA90K is a major ligand for galectin-3, which does mediate cellular adhesion (Sasaki *et al*., 1998; Ulmer *et al*., 2006), we examined the effect of TAA90K on galectin-3 mediated cellular adhesion. As shown in Figure 5.23, HT-29 cells adhered to galectin-3. However, the adhesion of HT-29 cells to galectin-3 was significantly inhibited in the presence of high amounts of TAA90K (5 μg/well) (P<0.0001). Surprisingly, as the amount of TAA90K relative to galectin-3 was reduced, galectin-3 mediated cell binding was enhanced with maximal cell adhesion detected in the presence of 8 ng TAA90K. This level of cell binding exceeded that seen with galectin-3 alone (P<0.0001). Thus, at high concentration, TAA90K exhibited anti-adhesive properties by interfering with galectin-3-mediated cell binding, however at low concentrations, it enhanced cell binding to galectin-3. When we examined the effect of TAA90K on cellular adhesion to other ECM proteins including laminin-1, laminin-10, fibronectin and collagen I, we observed no effect (data not shown).

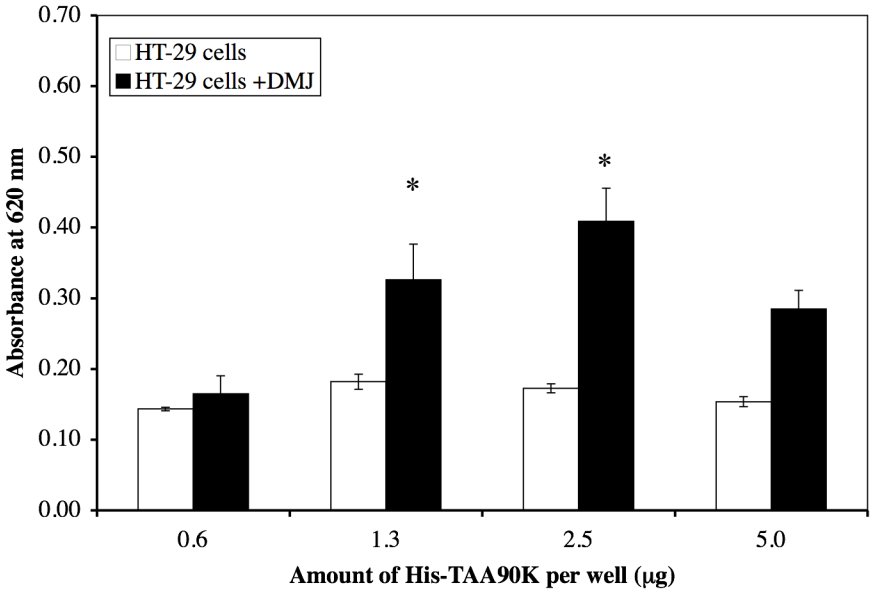


Figure 5.22: Adhesion assay to examine the effect of DMJ treatment on HT-29 adhesion to bacterially expressed TAA90K. HT29 cells (white bar) or HT29 cells pre-treated with DMJ (black bar) were incubated in wells coated with recombinant His-TAA90K purified from recombinant BL21 bacteria. Non-adherent cells were washed away and the remaining cells were fixed and stained. The adhesion of cells was detected spectrophotometrically at 620 nm. The mean and standard deviation of triplicate samples are shown. The asterisk indicates statistical significance with a *P*-value less than 0.05.

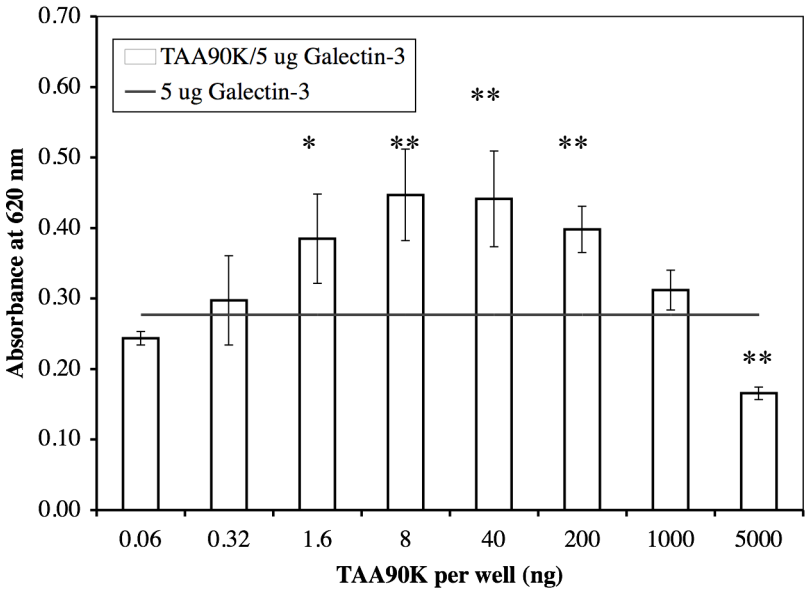


Figure 5.23: The effect of TAA90K on HT-29 cell adhesion to galectin-3. Wells coated with 5 μg galectin-3 were incubated with increasing amounts of TAA90K followed by addition of 10^5 HT-29 cells. The horizontal line running through the chart corresponds to the amount of HT-29 binding to galectin-3 alone. The mean and standard deviation of three separate experiments done in triplicate (n=9) are shown. The asterisks indicate statistically significant difference in cell binding compared to that observed with 5 μg galectin-3 alone (*P<0.005; **P<0.0001).

5.3.3.3 Effect of TAA90K on cell migration and invasion

Loss of cell-cell and cell-ECM interactions are important stages in the progression of colorectal cancer, enabling cancer cells to migrate and invade through the ECM during the metastatic process (Bracke *et al*., 1996; Giancotti and Ruoslahti, 1999). Galectin-3 has been implicated in modulating cell adhesion to ECM proteins as well as enhancing motility and invasion of cancer cells. For example, breast cancer cell lines that have been transfected with galectin-3 exhibited enhanced adhesion to ECM proteins including laminin and collagen IV (Warfield *et al.,* 1997). In addition, cells exhibited enhanced invasion through Matrigel in *in vitro* invasion assays, which was associated with an increased expression of $\alpha 6\beta 1$ integrin (Warfield *et al.,* 1997). Furthermore, increased expression of galectin-3 in poorly metastatic cell lines resulted in enhanced metastatic potential (Raz *et al.,* 1990). We have shown that TAA90K can behave as an anti-adhesive protein, inhibiting adhesion of HT-29 cells to galectin-3 when present at relatively high concentration. This suggested that TAA90K interferes in cell-galectin-3 interactions, thereby contributing to altered tumor cell migration and/or invasion, two important aspects of the metastatic process. We also observed that addition of TAA90K to HT-29 cells resulted in enhanced extracellular expression of proMMP-7 which could potentially lead to enhanced invasion of these cells.

In an attempt to elucidate the role of TAA90K in cellular migration and invasion, we performed cellular migration assays with HT-29 and SW620 cells. Briefly, cells were added to the upper chamber and allowed to migrate through pores in the polycarbonate filter. Cells that adhered to the underside of the filter were stained and quantitated. As shown in Figure 5.24, HT-29 cells migrated towards collagen I coated on the underside of a polycarbonate filter and SW620 cells migrated towards laminin-10 and fibronectin. However, neither cell line migrated towards TAA90K. Furthermore, addition of TAA90K to the upper chamber or to the bottom chamber had no effect on cellular migration towards these extracellular proteins.

Cellular invasion assays were employed to examine the effect of TAA90K on the invasion of HT-29 cells. In these experiments, increasing amounts of TAA90K or galectin-3 were pre-incubated with the reconstituted extracellular matrix (Matrigel) coated on the top of the polycarbonate filter of the inserts. The pre-incubation of

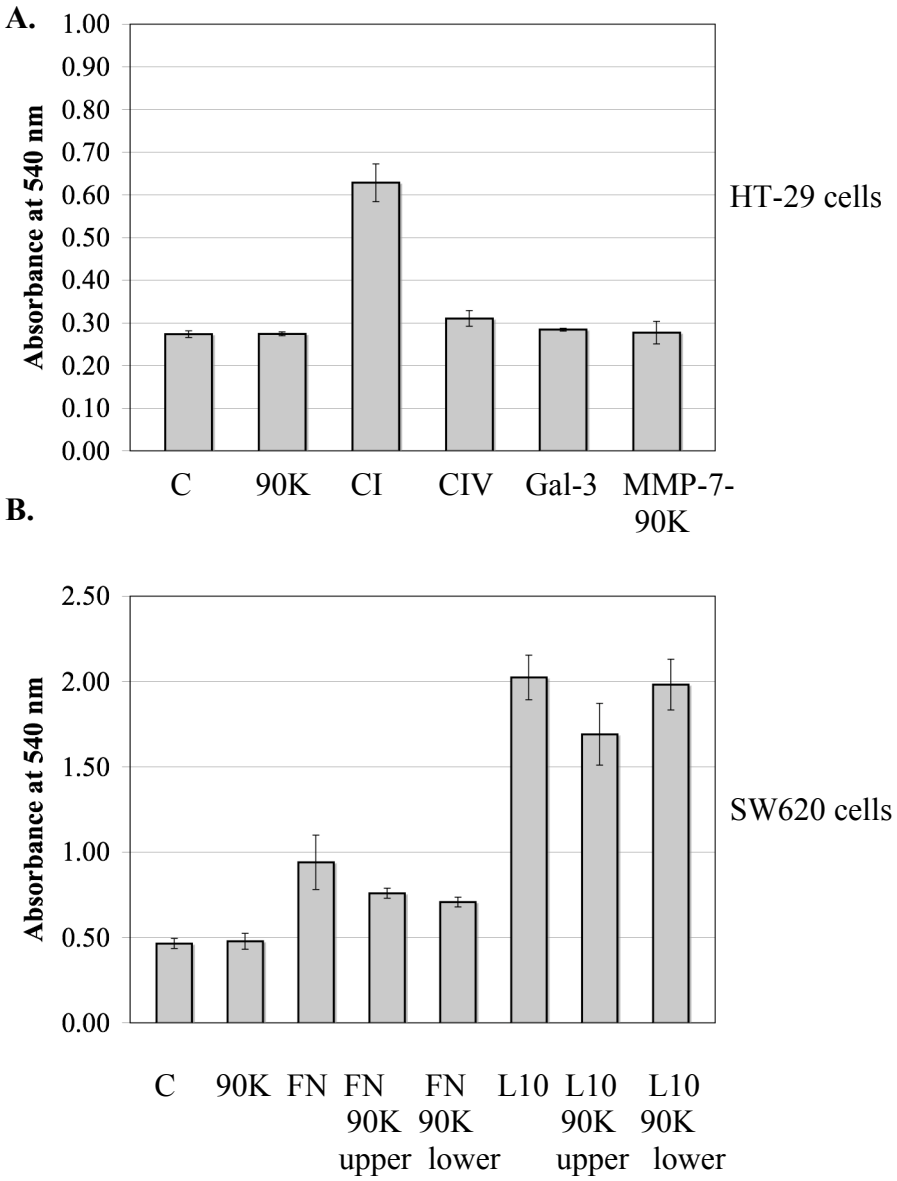


Figure 5.24 Migration assays examining the effect of TAA90K and extracellular proteins on cellular migration. Panel A: Migration of HT-29 cells towards no protein (C) or 5 μg each of collagen I (CI), collagen IV (CIV), TAA90K (90K), galectin-3 (Gal-3) or MMP-7 cleaved TAA90K (MMP-7- 90K) coated on the underside of the inserts. **Panel B** Migration of SW620 cells towards no protein (C) or 5 μg each of TAA90K (90K), fibronectin (FN) or laminin-10 (L-10) coated on the underside of the inserts. In some experiments where fibronectin and laminin-10 were coated on the underside of filters, 5 μg of TAA90K was either added with the cells to the upper chambers (FN 90K upper or L-10 90K upper) or to the bottom chambers (FN 90K lower or L-10 90K lower). Cells that migrated to the underside of the filter were stained with MTT and extracted with DMSO. Absorbances were read at 540 nm. The mean of triplicate experiments is shown and the error bars represent standard deviation.

TAA90K or galectin-3 did not effect the invasion of HT-29 cells through the reconstituted ECM layer, nor did adding TAA90K or galectin-3 in combination with the cells to the inserts. When we examined the affect of adding galectin-3 to the upper chamber in combination with different concentrations of TAA90K, no effect on the invasion of HT-29 cells through the Matrigel was observed (Figure 5.25).

5.3.3.4 The role of cleaved TAA90K in cell adhesion, migration and invasion

Although it appeared that intact TAA90K did not have a direct effect on cell adhesion, migration or invasion, we examined the effect of MMP-7-cleaved TAA90K in these functional assays. Previous studies have shown that cleavage of Fas-ligand or osteopontin by MMP-7 results in an altered function of the cleaved substrate compared to the intact proteins (Powell *et al*., 1999; Agnihotri *et al.,* 2001). To this end, we observed that MMP-7-cleaved TAA90K, similar to intact TAA90K, did not mediate adhesion, migration or invasion of HT-29 cells (Figure 5.24 and 5.25). However, MMP-7-cleaved TAA90K, like intact TAA90K, still modulated galectin-3 mediated cellular adhesion (Figure 5.26). Low concentrations of cleaved TAA90K enhanced galectin-3-mediated cell adhesion, as we showed previously for intact TAA90K (Ulmer *et al.,* 2006). This is not surprising because cleaved TAA90K bound well to galectin-3 in solid-phase binding assays (data not shown).

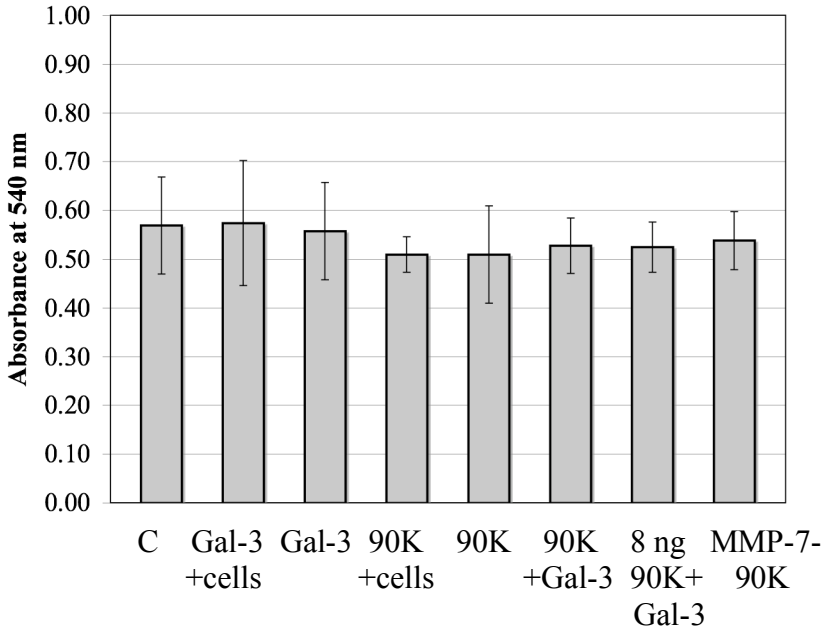


Figure 5.25 Invasion assay to examine the effect of TAA90K and galectin-3 on invasion of HT-29 cells. HT-29 cells were added to inserts coated with Matrigel (C) or Matrigel pre-incubated with 5 μg each of galectin-3 (Gal-3), TAA90K (90K), MMP-7-cleaved TAA90K (MMP-7-90K) or 5μg galectin-3 mixed with either 5 μg TAA90K (90K+Gal-3) or 8 ng TAA90K (8ng 90K+Gal-3). In some wells, HT-29 cells were added to the inserts in combination with either 5 μg galectin-3 (Gal-3+cells) or 5 μg TAA90K (90K+cells). Cells that invaded through the layer of Matrigel were stained with MTT and extracted with DMSO. Absorbances were read at 540 nm. The mean of experiments performed in triplicate is shown and the error bars represent standard deviation.

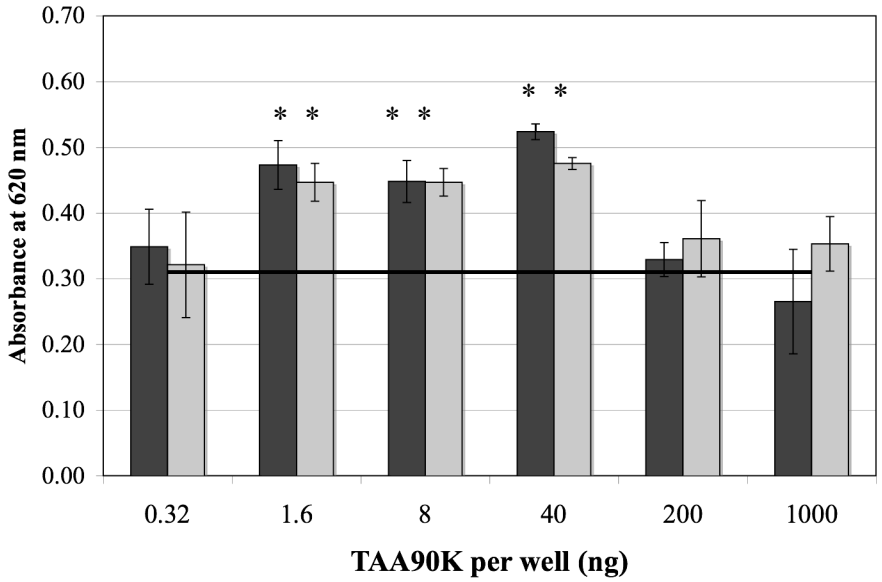


Figure 5.26 Adhesion assay to compare the effect of intact TAA90K and MMP-7 cleaved TAA90K on galectin-3-mediated HT-29 cell adhesion. Wells coated with 5 μg galectin-3 were incubated with increasing amounts of either intact TAA90K (black) or MMP-7-cleaved TAA90K (grey) followed by addition of 10^5 HT-29 cells. The horizontal line running through the chart corresponds to the amount of HT-29 binding to galectin-3 alone. The mean of an experiment done in triplicate is shown and the error bars represent standard deviation. The asterisk indicates a statistical significance for the effect of intact or cleaved TAA90K compared to galectin-3 alone with $P<0.05$.

6.0 DISCUSSION

In this study we have undertaken to examine the expression and possible role of TAA90K in human colorectal cancer. Previous studies have identified elevated levels of TAA90K expression in numerous types of cancer and shown a correlation between TAA90K expression and cancer progression (Marchetti *et al*., 2002; Ozaki *et al*., 2002; Singhal *et al*., 2003; Koopman *et al*., 2004). In addition, TAA90K was previously identified as a carrier of β1-6 branched N-linked oligosaccharides, cancer-associated carbohydrates associated with enhanced metastatic potential (Laferté and Loh, 1992; Ochwat *et al*., 2004; Kim *et al*., 2006). Thus, the examination of TAA90K expression and the contribution of complex carbohydrates to its function may enhance our understanding of its potential role in colorectal cancer initiation and progression.

6.1 Expression of TAA90K in colorectal cancer

A number of studies have examined the expression of TAA90K in cancer. For example, immunohistochemical analysis of TAA90K expression by non-small cell lung carcinoma (NSCLC) tumors revealed elevated levels that correlated with metastasis as well as decreased disease-free and overall survival (Marchetti *et al*., 2002). Other studies identified elevated TAA90K in the serum of patients suffering from breast, colon, stomach, ovarian and lung cancer (Linsley *et al*., 1986; Natoli *et al.,* 1993; Ullrich *et al*., 1994; Jallal *et al.,* 1995; Iacobelli *et al.,* 1986, 1993, 1994).

Our immunohistochemical analysis of human colorectal tumors revealed elevated levels of intracellular and luminal expression of TAA90K in all of the colon tumors analyzed compared to normal colonic mucosa (Ulmer *et al*., 2006). These findings suggested that TAA90K is a marker of cancer-associated changes within the colonic mucosa. This is likely due to enhanced transcriptional activation of the gene for TAA90K as a result of genetic alterations that result during carcinogenesis (Vogelstein and Kinzler, 2004).

A number of immunohistochemical staining patterns were observed, revealing that TAA90K is localized intracellularly, at the luminal surface of colon cells or in

some cases the basolateral surface. These staining patterns may indicate distinct functions of TAA90K in colorectal cancer depending on its sub-cellular localization. For example, in some early-stage Duke's A tumors, TAA90K was localized at the basolateral surface of colon cancer cells consistent with its ability to bind to extracellular matrix proteins such as laminins and collagens (Sasaki *et al*., 1998; Ulmer *et al.,* 2006). The localization of TAA90K at the basolateral surface of early-stage tumors may depict the localization of TAA90K in normal colonic mucosa. Although we were unable to detect TAA90K expression in normal colon, this is likely the result of the sensitivity of the immunohistochemical detection as other studies have revealed the presence of TAA90K mRNA in normal epithelial cells, including colon, by Northern blot analyses (Ullrich *et al.,* 1994).

Interestingly, increased expression of galectin-3 was also shown to correlate with poor prognosis in patients with colorectal cancer (Endo *et al*., 2006). In fact, galectin-3 expression in colon tumors sections was localized to the underlying stroma of the colonic mucosa (Greco *et al*., 2004), suggesting that TAA90K and galectin-3 may interact at the basolateral surface of cells to modulate cell-ECM contacts. In addition to its interaction with galectin-3, we identified MMP-7 as a ligand for TAA90K binding. Previous work implicated elevated MMP-7 expression in enhanced tumorigenesis and invasion in a number of cancers, including colorectal cancer (reviewed in McCawley and Matrisian, 2001). Immunohistochemical analysis of human gastric carcinoma revealed elevated levels of proMMP-7 expression at the basolateral surface of cancer cells (Yamashita *et al.,* 1998). These results would suggest that TAA90K may function by interacting with MMP-7 in the ECM, which may result in remodeling of the ECM and altered tumor cell-ECM interactions.

Elevated levels of granular, intracellular staining of TAA90K were observed in most colon tumors analyzed, consistent with its localization to secretory vesicles (Ulmer *et al*., 2006). Enhanced luminal expression of TAA90K was also observed in a number of tumor sections and is likely the result of enhanced secretion of TAA90K from the cancer cells. The luminal accumulation of TAA90K in colon tumors, a glycoprotein modified by β1-6 branched N-linked oligosaccharides (Laferté and Loh, 1992), suggested that TAA90K contributes to colon cancer progression and/or metastatic

disease. These results are supported by studies by Fernandes *et al*. (1991) that revealed elevated granular staining as well as luminal expression of glycoproteins bearing β1-6 branched N-linked oligosaccharides in human colon tumors, as detected by L-PHA. Significantly, the level of L-PHA staining of colon tumors was correlated with metastatic disease.

The elevated luminal expression of glycoproteins bearing β1-6 branched N-linked oligosaccharides, and potentially poly-N-acetyllactosamine repeats, would be expected to provide ligands for galectin-3 (van den Eijnden *et al.,* 1988; reviewed in Hughes, 2001). In fact, results have shown that in addition to being localized to the stroma in colon tumors, galectin-3 expression was also observed in luminal secretions (Greco *et al*., 2004). These findings suggest that TAA90K and galectin-3 may interact extracellularly, resulting in altered cell signaling events at the apical surface of colon tumor cells.

In addition to its potential interaction with galectin-3 at the apical surface, TAA90K may also interact with MMP-7 in luminal secretions. In support of this, immunohistochemical analysis of human gastric carcinoma revealed elevated levels of proMMP-7 expression at the apical surface of cancer cells in addition to enhanced basolateral expression (Yamashita *et al.,* 1998). MMP-7 expression was also observed in the lumen of dysplastic colon tumors isolated from mice (Wilson *et al.,* 1997). In addition, the identification of substrates for MMP-7 that are localized to both the apical and basolateral surfaces would suggest that MMP-7 may function at both locations (McCawley and Matrisian, 2001). We demonstrated that TAA90K is localized at both the basolateral and luminal surfaces of colon tumors, in addition to being a substrate for MMP-7 activity. Thus, TAA90K and MMP-7 may function at the apical surface of tumor cells resulting in altered cellular signaling events. In support of this, elevated apical secretion of MMP-7 by polarized Madin-Darby canine kidney (MDCK) cells was implicated in elevated cellular growth and proliferation (Harrell *et al*., 2005).

Immunohistochemical analysis of human colon tumors also revealed elevated expression of TAA90K compared to normal mucosa. These results were confirmed by Western blotting analysis following immunoprecipitation of detergent lysates of colon tumors and their adjacent normal mucosa with a TAA90K-specific monoclonal

antibody. Western blotting analysis revealed differences in the apparent molecular mass of TAA90K immunoprecipitated from different colon tumors (Ulmer *et al*., 2006). These results are consistent with previous findings that identified variations in the molecular mass of TAA90K isolated from different human colon cancer cell lines. This variability was shown to be the result of cell-specific differences in glycosylation (Laferté and Loh, 1992). These results suggested that human colon cancer cell lines are an adequate representation of human colon tumors and thus, are useful for *in vitro* studies. To this end, we examined the secretion of TAA90K into the conditioned medium of human colon cancer cell lines. ELISA analysis employing a TAA90K-specific monoclonal antibody revealed variable levels of TAA90K secretion by different colon cancer cell lines, consistent with the variable levels of TAA90K expression detected in different human colon tumours. Interestingly, the colon cancer cell lines that were established from metastases (KM20C and SW620) expressed the highest levels of TAA90K, suggesting a role in the development of lymph node and distant metastases in patients with colon cancer.

6.2 Purification of TAA90K

Although the accumulation of TAA90K in a number of cancers has been associated with poor outcome (Marchetti *et al*., 2002; Ozaki *et al*., 2002; Singhal *et al*., 2003), the mechanisms of TAA90K function have yet to be elucidated. Since TAA90K can be localized in the ECM, interact with ECM proteins as well as mediate cell-cell interactions, TAA90K may play a role in colon cancer progression (Inohara *et al*., 1996; Sasaki *et al*., 1998). In order to elucidate the role of TAA90K in colon cancer, we undertook to purify TAA90K from human colon cancer cells for use in functional assays. We employed a number of chromatographic methods including lectin-affinity chromatography and ion-exchange chromatography to isolate TAA90K or TAA90K-His from the human colon cancer cell line HT-29 infected with recombinant virus expressing TAA90K or TAA90K-His, respectively. The purity and identity of the 105 kDa protein was confirmed by mass spectrometry (Ulmer *et al*., 2006).

6.3 Functional experiments with TAA90K

6.3.1 Interaction of TAA90K with ECM proteins

We confirmed previous findings that TAA90K binds to ECM proteins such as fibronectin, collagen IV, laminin-1 and to a lesser extent collagen I (Sasaki *et al*., 1998; Ulmer *et al*., 2006). In addition, we demonstrated TAA90K binding to laminin-5 and -10. In an attempt to identify the domains of TAA90K that are required to mediate binding to ECM proteins, Hellstern *et al.* (2002) carried out solid-phase binding assays with TAA90K constructs composed of different combinations of putative domains (Figure 2.15). For example, a recombinant TAA90K consisting of putative domains 3 and 4 exhibited minimal binding to fibronectin, whereas, a construct consisting of domains 2, 3 and 4 did bind fibronectin. These results suggested that domain 2 may contain a binding site for fibronectin. Alternatively, the absence of domain 2 may result in altered TAA90K conformation, rendering the fibronectin binding site inaccessible. Interestingly, we demonstrated that cleavage of TAA90K in the linker region (between domains 3 and 4) by MMP-7 resulted in reduced binding to fibronectin. Thus, this cleavage may result in altered conformation of TAA90K monomers, limiting access to the fibronectin binding domain. These results, in addition to the observed basolateral localization of TAA90K in some early stage Duke's A colon tumors (Ulmer *et al*., 2006) and its expression in the ECM of normal tissues (Sasaki *et al*., 1998), would suggest that TAA90K may function in normal tissues and early cancers by being deposited in the ECM and by binding to extracellular proteins.

6.3.2 TAA90K and cellular adhesion

In addition to examining the interaction of TAA90K with ECM proteins, we also examined the ability of TAA90K to mediate the adhesion of cells. Previous work revealed that TAA90K isolated from other cell types was able to mediate the adhesion of A431 epidermoid carcinoma cells as well as HBL-100 breast epithelial cells in a β1-integrin-dependent manner (Sasaki *et al*., 1998). For these studies, recombinant TAA90K was purified from EBNA-293 human embryonic kidney cells transfected with the cDNA encoding TAA90K. The purification protocol consisted of ammonium sulfate

precipitation and gel exclusion chromatography of the conditioned medium and yielded TAA90K of 92 kDa (Sasaki *et al*., 1998).

In contrast, TAA90K isolated from HT-29 cells was not able to mediate the adhesion of seven colon cancer cell lines nor was it able to mediate the adhesion of a number of normal cell lines including HBL-100 cells. These results suggested that HT-29-derived TAA90K is modified by cell-type-specific carbohydrates which convert it to an anti-adhesive substrate. This is supported by the apparent molecular weight difference between the 92 kDa TAA90K isolated from embryonic kidney cells compared to the 105 kDa TAA90K isolated from HT-29 cells. Furthermore, HT-29 cells treated with 1-deoxymannojirimycin, which inhibits the conversion of high-mannose type to complex-type N-glycans, adhered better to bacterially expressed TAA90K, which lacks carbohydrate modifications, than to fully glycosylated TAA90K. Thus, the presence of carbohydrate moieties on both cell surface receptors and their glycosylated ligands may be important in mediating TAA90K-cellular interactions. The potential anti-adhesive function of TAA90K-derived from human colon cancer cells suggested that it may interfere with specific tumor cell-ECM contacts, resulting in enhanced tumor cell motility associated with a more invasive phenotype. Although we did not observe an effect of TAA90K on cellular migration using *in vitro* migration assays, the effects mediated by TAA90K may not have been adequately measured employing this type of functional assay or may require co-stimulatory molecules.

Another possibility for the observed differences in TAA90K-mediated cellular adhesion is the purification strategies employed to isolate TAA90K from different cellular sources. For example, we initially purified TAA90K from HT-29 cells employing either ammonium sulfate precipitation or lectin-affinity chromatography followed by anion-exchange and gel exclusion chromatography. Although we did observe cellular adhesion to this preparation of TAA90K, this adhesion was due to fibronectin contamination (data not shown). When fibronectin was later removed by cation-exchange chromatography, our TAA90K preparations no longer mediated HT-29 cellular adhesion. In light of these findings, TAA90K preparations isolated from other cell types (Sasaki *et al*., 1998; Hellstern *et al.,* 2001) may have been contaminated with fibronectin. Although these studies demonstrated β1-integrin-dependent cellular

adhesion to their TAA90K preparations, they did not observe cellular adhesion to recombinant TAA90K consisting only of domains 3 and 4. This form of TAA90K which contained a histidine tag, was purified from the medium of transfected EBNA-293 cells by gel-exclusion and metal chelation affinity chromatography (Hellstern *et al.,* 2002). This form of TAA90K also failed to bind to fibronectin, suggesting a conformational change from wild-type TAA90K resulting in reduced accessibility to cellular receptors and fibronectin binding domains. Alternatively, recombinant TAA90K containing domains 3 and 4 was isolated employing different chromatographic techniques compared to initial experiments with wild-type TAA90K, which removed contaminating proteins such as fibronectin capable of mediating cell adhesion.

6.3.3 TAA90K and galectins

Elevated levels of galectin-1, galectin-3 and TAA90K have been associated with malignant cell transformation and cancer progression (reviewed in Grassadonia *et al*., 2004). For example, increased expression of galectin-1 and/or galectin-3 has been correlated with the malignant potential of a number of cancers including colorectal (Schoeppner *et al*., 1995; Sanjuan *et al*., 1997), glioma (Camby *et al*., 2001) and gastric carcinoma (Lotan *et al*., 1994). As previously mentioned, elevated serum levels of TAA90K correlate with progression and poor prognosis in a number of cancers including breast, colon, stomach, ovary and lung (Marchetti *et al*., 2002; Ozaki *et al*., 2002; Singhal *et al*., 2003; Koopman *et al*., 2004). In light of these findings, we examined the interaction between TAA90K isolated from a human colon cancer cell line and galectins-1 and -3, in an attempt to elucidate their roles in colon cancer progression.

Our studies revealed that TAA90K binds to galectin-3 in a carbohydrate dependent manner, as expected for glycoproteins modified with poly-N-acetyllactosamine structures (Laferté and Loh, 1992; reviewed in Hughes, 2001). However, in contrast to TAA90K isolated from fibroblasts (Tinara *et al*., 2001) TAA90K purified from HT-29 cells bound poorly to galectin-1 (Ulmer *et al*., 2006). The difference in binding to galectin-1 was likely due to cell-specific differences in

glycosylation and the selectivity of galectin binding for specific carbohydrate structures (Ahmad *et al*., 2002). In this regard, HT-29 cells were derived from a patient with histo-blood group A status consistent with previous work in our laboratory showing that TAA90K isolated from these cells binds to a monoclonal antibody specific for type 2 chain blood group A antigens (data not shown; Prokophishyn *et al*., 1999). Galectin-3 binds well to blood group A carbohydrate antigens which are present on HT-29-derived TAA90K, however, galectin-1 binding was shown to be reduced in the presence of these carbohydrate structures (Sparrow *et al*., 1987; Ahmad *et al.,* 2002). Differences in binding of galectin-1 and galectin-3 to TAA90K were previously identified by Tinari *et al.* (2001) who determined that these galectins bind to different carbohydrate structures on TAA90K, as they do not compete with each other for binding. In summary, TAA90K isolated from HT-29 cells contains carbohydrate structures that are binding sites for galectin-3, but not galectin-1, suggesting that TAA90K may mediate its function in colon cancer in part by interacting with galectin-3.

Since we were unable to observe TAA90K-mediated cellular adhesion, we examined the effect of TAA90K binding to galectin-3-mediated cellular adhesion. Previous studies have shown that galectin-3 mediates adhesion of a number of types of cells (André *et al*., 1999). Our studies demonstrated that galectin-3 can mediate adhesion of HT-29 cells (Ulmer *et al*., 2006). Studies examining cellular aggregation determined that the addition of TAA90K to melanoma cells expressing galectin-3 on the cell surface resulted in enhanced homotypic interactions, suggesting that TAA90K and galectin-3 interact on the cell surface to promote cell-cell adhesions (Inohara *et al*., 1996). These interactions may be involved in enhancing the metastatic process by forming stable tumor emboli, which are more likely to survive in the vasculature upon metastasis. In support of a role for TAA90K and galectin-3 in mediating homotypic interactions during embolization, recent studies have shown that elevated plasma levels of TAA90K in patients with colorectal cancer correlated with cancer cell surface expression of galectin-3 (Greco *et al*., 2004).

We demonstrated that TAA90K modulates galectin-3-mediated adhesion of HT-29 cells (Ulmer *et al*., 2006). At high concentration, TAA90K inhibited cellular adhesion to galectin-3, but at lower concentrations it enhanced cell binding. Other

studies have examined cell-mediated effects of galectin-3 to find that sub-micromolar amounts of galectin-3 inhibit cellular adhesion and high concentrations promote adhesion (Inohara *et al.,* 1996). These observations may be the result of enhanced galectin-3 oligomerization at high concentrations, which may promote cellular adhesion through the ability to form cross-links between cells and ECM proteins (Ahmad *et al*., 2004). Our results suggested that high levels of TAA90K bind galectin-3 preventing it from accessing cell surface receptors. Alternatively, reduced levels of TAA90K may lead to enhanced galectin-3 mediated cellular adhesion possibly through the enhancement of galectin-3 oligomerization. Thus, the ratio of TAA90K and galectin-3 secreted by colon cancer cells may play a role in colon cancer progression and metastasis by modulating tumor cell adhesion. Results demonstrating that both TAA90K and galectin-3 are localized to the stroma in some colorectal tumors, supports their roles in the modulation of tumor cell adhesion to the ECM (Greco *et al.*, 2004; Ulmer *et al*., 2006).

In addition to their localization to the stroma of colon tumors, enhanced luminal expression of both galectin-3 and TAA90K has also been observed (Greco *et al.*, 2004; Ulmer *et al*., 2006). The extracellular accumulation of TAA90K and galectin-3 at the apical surface of colon cancer cells suggests a role in cellular signaling events. Since both TAA90K and galectin-3 have been implicated in immunomodulation, including stimulation of pro-inflammatory responses and modulation of T cell responses (Powell *et al*., 1995;Trahey and Weissman, 1999; Rabinovich *et al*., 2002; Nakahara *et al*., 2005), their potential luminal interaction may be involved in modulating host immune responses to promote colorectal cancer progression.

6.3.4 TAA90K and MMPs

Previous studies have revealed that MMPs are the main proteinases involved in the invasion of colorectal carcinomas. Specific MMPs that have been implicated in colorectal cancer include MMP-1, -2, -3, -7, -9, -12, -13 and MT1-MMP (reviewed in Zucker and Vacirca, 2004). Although MMPs play a role in colorectal cancer, MMP-7 appears to have the most significant effect not only on colorectal tumorigenesis, but also in invasion and metastasis (reviewed in Wagenaar-Miller *et al*., 2004). The expression

of MMP-7 is observed at early stages of tumor progression and has been implicated in enhancing cellular proliferation as well as the progression from adenoma to invasive carcinoma (reviewed in Wagenaar-Miller *et al*., 2004; Harrell *et al*., 2005). MMP-7 expression was observed to be localized in the lumen of dysplastic colon tumors isolated from mice (Wilson *et al.,* 1997). Other studies have revealed that MMP-7 is expressed by 90% of colonic adenocarcinomas and its expression is correlated with the presence of lymph node as well as distant metastases (Newell *et al*., 1994).

We examined the expression of proMMP-7 and active MMP-7 in a number of human colon cancer cell lines. We observed variable levels of proMMP-7 expression with the various cell lines, with HT-29 and KM20C cells expressing the highest levels of proMMP-7. We observed minimal amounts of active MMP-7 expression in any of the cell lines tested, but these results are likely due to the low sensitivity of the ELISA assay used. Our results are consistent with previous studies, which failed to detect active MMP-7 in the conditioned medium of HCA-7 and Caco-2 human colon cancer cells or from MMP-7 transfected Madine-Darby canine kidney (MDCK) cells. However, in a recent study, a more sensitive fluorometric assay was successful in detecting MMP-7 enzyme activity in the conditioned medium of cells (Harrell *et al.,* 2005).

In addition to enhancing the invasion of cancer cells, MMPs have also been implicated in the recruitment of leukocytes to a site of injury during inflammation (reviewed in Coussens and Werb, 2002). Inflammation is a tightly regulated process controlled by both anti-inflammatory and pro-inflammatory responses (reviewed in Coussens and Werb, 2002). Inflammation commonly results in response to wound healing or infection and is associated with the secretion of pro-inflammatory cytokines. After loss of anti-inflammatory signals, chronic inflammation can result and is characterized by elevated expression of pro-inflammatory cytokines, which enhance tissue remodeling by stimulating DNA replication and cellular proliferation. These events have been associated with the initiation of cancer (reviewed in Coussens and Werb, 2002). For example, inflammatory bowel diseases such as ulcerative colitis and Crohn's disease are associated with elevated levels of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-8 from mononuclear cells (Neurath *et al*., 1998).

The expression of these cytokines is mediated by NFκB, which enhances the transcription of the genes for these cytokines (Neurath *et al*., 1998). In addition, patients with inflammatory bowel disease exhibit increased levels of MMP-1, -3, -7 and -14 (Newell *et al.* 1994). Thus, individuals with inflammatory bowel diseases are at elevated risk for colon cancer due to chronic inflammation (Campbell *et al*., 2001).

Interestingly, the expression of proMMP-7 in prostate cancer cells was shown to be regulated by the NFκB-dependent expression of cytokines including IL-1β and IL-6, leading to STAT3 activation (Maliner-Stratton *et al*., 2001). Furthermore, studies by Bair *et al*. (2006) revealed that the addition of purified TAA90K to prostate cancer cells resulted in the enhanced extracellular expression of proMMP-7 in an IL-6-dependent manner. Consistent with these findings, we showed that the addition of purified TAA90K to HT-29 cells was able to elevate the extracellular expression of proMMP-7. Thus, TAA90K may result in enhanced extracellular expression of proMMP-7 through a number of possible mechanisms. For example, TAA90K may interact with proMMP-7 extracellularly and prevent access by activating proteases, effectively increasing the half-life of proMMP-7 in the medium. TAA90K does not appear to increase the extracellular expression of proMMP-7 by binding to inhibitors of MMP activity, since TAA90K did not bind to TIMP-1, TIMP-2 or a_2-macroglobulin. Alternatively, TAA90K may enhance the extracellular expression of proMMP-7 by stimulating the secretion of IL-6. Previous studies have shown that TAA90K is capable of stimulating the release of cytokines, including IL-6 (Ullrich *et al*., 1994; Powell *et al*., 1995). In addition to being able to stimulate the release of IL-6, we demonstrated in solid-phase binding assays that TAA90K is also able to interact directly with both IL-6 and IL-1β. These results suggested that TAA90K may stimulate pro-inflammatory responses by binding to cytokines as well as stimulating their release. The binding of TAA90K to cytokines extracellularly may result in enhanced presentation of the cytokines to their cell surface receptors. In support of the role of TAA90K in cytokine-mediated cellular signaling events, addition of MMP-7-cleaved TAA90K, which exhibited reduced binding to both IL-6 and IL-1β, resulted in reduced extracellular expression of proMMP-7 from HT-29 cells. Thus, TAA90K may enhance extracellular expression of proMMP-7 by modulating pro-inflammatory responses, which have been previously implicated in the

initiation of colon cancer (reviewed in Coussens and Werb, 2002; Campbell *et al*., 2001).

Although we observed enhanced extracellular expression of proMMP-7 from HT-29 cells grown in the presence of TAA90K, we did not observe an effect of TAA90K on the migration or invasion of these cells. TAA90K may function by preventing activation of proMMP-7, resulting in elevated extracellular accumulation of proMMP-7 and either no effect on cellular invasion or decreased cellular invasion. Alternatively, these results suggest that TAA90K and MMP-7 may function during early stages of colon carcinogenesis and may not be directly involved in the invasive process. In support of this, MMP-7 has been shown to play an important role in invasion of colon carcinogenesis, however, it has also been implicated in earlier processes involving enhanced cellular proliferation and tumorigenesis (reviewed in Wagenaar-Miller *et al*., 2004; Harrell *et al*., 2005). In this regard, elevated apical expression of active MMP-7 resulted in enhanced growth and cellular proliferation of MDCK cells expressing human MMP-7 (Harrell *et al*., 2005). Furthermore, TAA90K-enhanced extracellular expression of proMMP-7 by prostate cells was shown to be dependent on IL-6 resulting in the activation of STAT3 (Maliner-Stratton *et al*., 2001; Bair *et al.,* 2006). Activation of signal transducer and activator of transcription 3 (STAT3) has recently been implicated in enhanced cell proliferation and tumor growth in colon cancer (Corvinus *et al.,* 2005). Thus, TAA90K-enhanced proMMP-7 expression may not play a role in invasion, but rather, be involved in enhancing earlier stages of colon carcinogenesis including cellular proliferation and tumorigenesis.

It has been well established that MMPs function extracellularly by cleaving a number of ECM and non-ECM proteins such as galectin-3, FasL and osteopontin (Ochieng *et al*., 1994; Powell *et al*., 1999; Agnihotri *et al*., 2001). Cleavage of specific substrates by MMPs can generate cleavage products with altered function compared to the intact protein. For example, cleavage of galectin-3 by MMP-2 and -9 resulted in altered binding to glycoconjugate ligands (Ochieng *et al*., 1994). In addition, the cleavage of osteopontin by MMP-7 resulted in the generation of cleavage products capable of enhancing cellular migration (Agnihotri *et al*., 2001). Previous studies have revealed that TAA90K is susceptible to proteolysis by plasmin and trypsin (Hellstern *et*

al., 2002) suggesting that TAA90K may be a substrate for proteolytic cleavage by other proteases including MMPs. In support of this, recent work by Canals *et al.* (2006) identified TAA90K as a putative substrate for the metalloproteinase ADAMTS1. In addition, our studies revealed that TAA90K interacts directly with proMMP-7 as well as active MMP-7. On the other hand, little or no interactions were observed with other MMPs including MMP-1, proMMP-2, active MMP-2, proMMP-9 or active MMP-9.

In addition to binding to MMP-7, our data revealed that TAA90K was cleaved by MMP-2, -7 and -9, MMPs which have been implicated in poor prognosis for patients with colorectal cancer (reviewed in Zucker and Vacirca, 2004). N- and/or C-terminal sequence analysis of the 79 and 22 kDa TAA90K fragments produced by MMP-7 cleavage revealed a major proteolytic cleavage site between amino acid residues Asp ^{452} and Tyr ^{453}at the junction between the linker region and putative domain 4 and near the plasmin and trypsin cleavage sites (Hellstern *et al*., 2002). These data suggested that this region of TAA90K is a protease-sensitive region exposed on the surface of TAA90K oligomers.

Cleavage of TAA90K by proteinases may result in the generation of TAA90K fragments with altered activity compared to the intact protein. In support of this, MMP-7 cleavage of TAA90K resulted in reduced affinity for fibronectin and laminin-10. Thus, cleavage by MMP-7 may prevent interactions between TAA90K cleavage products and fibronectin or laminin-10 in the ECM, allowing these proteins to be more accessible to their cell surface receptors. The reduced binding of MMP-7-cleaved TAA90K to fibronectin may also affect signaling events initiated by fibronectin binding to its $\alpha5\beta1$ integrin receptor. In this regard, studies examining the expression of MMP-13 in fibroblasts isolated from wild-type or CyCAP-null mice revealed that induction of MMP-13 expression by fibronectin fragments or IL-1β is reduced in CyCAP-null cells (Kong *et al*., 2004). These results suggested that CyCAP is involved in the regulation of fibronectin fragment-induced MMP-13 expression. Thus, intact TAA90K may interact with fibronectin and have a similar effect in the regulation of MMP expression in human cells. The altered affinity of MMP-7-cleaved TAA90K for fibronectin may be a regulatory mechanism involved in this pathway and may be modulated by altered binding of MMP-7-cleaved TAA90K to IL-6 and IL-1β, two cytokines implicated in

enhanced extracellular expression of proMMP-7 (Maliner-Stratton *et al.*, 2001). Since cleavage of TAA90K by MMP-7 results in reduced extracellular expression of proMMP-7, this may function as a negative feedback loop in the regulatory pathway that controls MMP-7 expression.

Collectively, these results suggest that TAA90K is a multi-adhesive protein that may function in the extracellular matrix or be involved in cellular signaling events at the apical surface of cells. TAA90K has been found localized at the basolateral membrane and was capable of interacting with extracellular proteins including laminins, collagens, fibronectin, MMPs and galectin-3, suggesting a structural role in the maintenance of the ECM. TAA90K was not able to mediate cellular adhesion directly, but it does modulate cellular adhesion mediated by galectin-3. The fact that TAA90K stimulates the extracellular expression of proMMP-7 by colon cancer cells suggests a potential role for TAA90K in the remodeling of the ECM during wound healing or invasion by carcinoma cells. The induction of proMMP-7 expression appears to be regulated by MMP-7-cleavage of TAA90K, which results in reduced interaction with cytokines and reduced extracellular expression of proMMP-7 by HT-29 cells.

In the colon tumors that we analyzed, the majority of TAA90K was localized in the lumen of the tissues, suggesting a role in cell signaling events. In support of this, previous studies have demonstrated luminal expression of galectin-3 and MMP-7 in colon tumors, suggesting extracellular co-localization with TAA90K and potential modulation of signaling events including inflammatory responses. The cleavage of TAA90K by MMPs such as MMP-7 may be involved the regulation of inflammatory responses in the colon. Thus, TAA90K, galectin-3 and MMP-7 may contribute to cancer progression by enhancing pro-inflammatory responses, which have been implicated in the development of cancer.

7.0 CONCLUSIONS

In addition to being a glycoprotein that is modified by cancer-associated carbohydrates, elevated TAA90K expression has been correlated with poor prognosis in a number of cancers. The results of this thesis have demonstrated elevated expression of TAA90K in human colon tumors, suggesting a role as a marker for neoplastic changes in the colon. TAA90K is a multi-functional protein capable of interacting with proteins in the ECM with the potential to modulate tumor cell adhesion and ECM remodeling. Furthermore, TAA90K may stimulate inflammatory responses in the colon resulting in elevated levels of IL-6 and MMP-7 secretion, which are risk factors for colon cancer.

Further analysis of the role of TAA90K in colon cancer progression will be required to elucidate the mechanisms involved in TAA90K-mediated enhanced extracellular expression of proMMP-7. In addition, examination of the effect of TAA90K and MMP-7 on tumorigenesis will be required.

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